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Dihaloethane and alpha-Naphthylisothiocyanate Toxicity.

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Glutathione, a ubiquitous cellular tripeptide, is recognized as the most abundant nonprotein thiol in most cells. Although there are many cellular functions presently recognized for glutathione, chemical detoxification and protection against activated oxygen species are considered paramount. The participation of glutathione in detoxification reactions typically relate to conjugation with toxic species limiting further interaction with cellular constituents and aiding excretion of the toxicant from the body. However, conjugation of certain xenobiotics with glutathione may actually result in the formation of a more toxic compound. This increased toxicity may be the direct result of glutathione conjugation or may relate to the formation of a glutathione conjugate that releases a reactive species upon further metabolism. In addition, glutathione conjugation may function in the transport of toxicant to tissues more susceptible to toxicity. This thesis details my investigation of the role of glutathione conjugation in the *in vitro* toxicity of 1,2-dihaloethanes and alpha-naphthylisothiocyanate. The use of freshly

isolated hepatocytes as a model system for study has allowed me to investigate the role of glutathione conjugation in the metabolism and toxicity of these hepatotoxins. My studies have demonstrated that hepatocytes utilize glutathione extensively in 1,2-dihaloethane and alpha-naphthylisothiocyanate metabolism. Glutathione conjugation to either of these agents can lead to extensive glutathione depletion and cell death. Glutathione conjugation with 1,2-dihaloethanes produces reactive sulfur half-mustards that can function directly as alkylating agents. This has been demonstrated for S-(2-chloroethyl)glutathione. My studies with alpha-naphthylisothiocyanate show that it's reversible conjugation with glutathione may promote hepatocellular glutathione depletion and biliary excretion of alpha-naphthylisothiocyanate. The relevance of these findings to the *in vivo* mechanism of action of these agents is discussed in the respective chapters.

Glutathione Conjugation as a Determinant in 1,2-
Dihaloethane and alpha-Naphthylisothiocyanate Toxicity

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Glutathione Conjugation as a Determinant in 1,2-Dihaloethane and alpha-Naphthylisothiocyanate Toxicity

INTRODUCTION

Glutathione: Function and Biological Significance

Glutathione is a tripeptide. The structure of which was established through chemical characterization and synthesis as L-gamma-glutamyl-L-cysteinylglycine (Hopkins, 1929, Kendall et al., 1930, Harington and Mead, 1935). This ubiquitous tripeptide has been identified in animals, plants and many microorganisms.

In animals, glutathione is thought to be present in all tissues at concentrations ranging from 0.5 to 12.0 mM (Kosower and Kosower, 1978). Table 1 lists some of the reported values for various tissues. The presence of an atypical peptide bond between the gamma-carboxylate of the glutamyl residue and the alpha-amino of the cysteinyl residue is thought to spare glutathione from degradation by the cellular peptidases. Protection from hydrolytic breakdown likely contributes to the accumulation of glutathione to such high intracellular concentrations.

The term "glutathione" will be used throughout this thesis to denote specifically the reduced form of the tripeptide. This form accounts for the majority of the glutathione equivalents within most cell types under normal conditions. However, other forms may be present including glutathione disulfide, glutathione-protein mixed disulfides, other glutathione-mixed disulfides and thioethers (Kosower and Kosower, 1978).

Table 1. Tissue Levels of Glutathione^a.

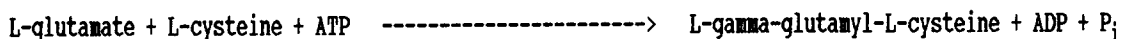
Tissue	Concentration (mM)
Erythrocytes	2.0 - 3.0
Leukocytes	3.5 - 5.0
Kidney (rat)	2.5
Liver (rat)	4.5 - 6.5
(mouse)	3.0
Lens	2.6 - 12.0
Nervous tissue	2.0 - 3.4
Stomach (rat) ^b	7.2 - 8.2

^aKosower and Kosower (1978), ^bBoyd et al. (1979).

The study of glutathione in the liver as a whole tissue as well as with isolated hepatocytes *in vitro* have demonstrated the existence of two distinct "pools" of intracellular glutathione (Jocelyn, 1975, Meredith and Reed, 1982, Tateishi and Higashi, 1978, Wahllander et al., 1979). The largest pool under normal conditions is the cytosolic pool which constitutes 85-90% of the total hepatocellular glutathione. This pool has a half-life of approximately 2.0 hours. The remaining 10-15% of the total glutathione is found in mitochondria and has an approximate half-life of 30 hours. It is uncertain if other tissues exhibit this same degree of glutathione compartmentalization. The half-life of glutathione may be tissue specific. Dependent upon it's rate of synthesis, breakdown, utilization, uptake and excretion. In addition, mitochondria are thought not to possess the enzymes necessary for glutathione synthesis. They acquire glutathione via uptake from the cytosol (Griffith and Meister, 1985).

The synthesis of glutathione is thought to occur in virtually all mammalian tissues. Glutathione is synthesized from its constituent amino acids in the cytosolic compartment of cells and involves two ATP-dependent enzymatic steps (see below) (Meister and Tate, 1976). gamma-Glutamylcysteine synthetase catalyzes the first step in glutathione synthesis in which glutamic acid and cysteine are linked through the atypical gamma-carbonyl-derived peptide bond. The addition of glycine to this dipeptide is then carried out by glutathione synthetase to yield glutathione. The availability of cysteine would appear to be the limiting factor in glutathione synthesis as the intracellular concentration of cysteine (0.1 - 0.3 mM) is much lower than that of the other two amino acids (Tateishi *et al.*, 1974). However, the availability of cysteine for glutathione biosynthesis in the liver can be greatly enhanced via the cystathionine pathway (Table 2) (Reed and Beatty, 1978, Reed and Orrenius, 1977). Glutathione synthesis can also be regulated by glutathione itself as it can inhibit the activity of gamma-glutamylcysteine synthetase (Richman, P. and Meister, A., 1975).

gamma-glutamylcysteine synthetase



glutathione synthetase

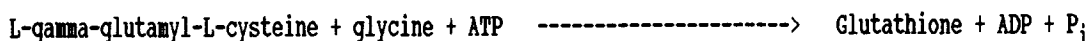


Table 2. Cystathionine Pathway^a: The Synthesis of Cysteine from Methionine and Serine.

Step	Reaction (Enzyme)
1	L-Methionine + ATP -----> S-Adenosyl-L-methionine + PP _i + P _i (S-Adenosylmethionine Synthetase)
2	S-Adenosylmethionine + R ^b -----> S-Adenosylhomocysteine + CH ₃ -R (Methyl Transferase)
3	S-Adenosylhomocysteine -----> L-Homocysteine + Adenosine (S-Adenosylhomocysteine Hydrolase)
4	L-Homocysteine + L-Serine -----> L-Cystathionine (Cystathione Synthase)
5	L-Cystathionine -----> L-Cysteine + alpha-Ketobutyrate + NH ₄ ⁺ (gamma-Cystathionase)

^aGreenberg (1975) ^bR = methyl group acceptor

Numerous roles have been identified for glutathione in animals (Table 3). It's role in cellular protection against reactive oxygen species, radiation generated free radicals and reactive xenobiotic metabolites are now well recognized (Moldeus and Quanguan, 1987, Reed, 1985, Reed and Meredith, 1984, Bump and Brown, 1990).

Biotransformation denotes the enzymatic metabolism of foreign compounds (xenobiotics) to more water soluble forms in an effort to promote their excretion from the body. This process has been divided into two separate parts. The first is referred to as Phase I metabolism and involves oxidation, reduction, or hydrolysis of the xenobiotic resulting in the addition or unmasking of functional groups to be used in Phase II metabolism. The principle action in Phase II metabolism is the conjugation of the Phase I metabolites with a water soluble moiety. A variety of different conjugation reactions can take place depending upon the nature of the Phase I metabolite. Glucuronidation, sulfation, and glutathione S-conjugation represent the major types of conjugation reactions that characterize the Phase II metabolic system. Glutathione is a very important participant in Phase II metabolism of a wide variety of xenobiotics (Chasseaud, 1979, Coles and Ketterer, 1990).

The conjugation of glutathione to xenobiotic metabolites may be either enzymatic or nonenzymatic depending upon the characteristics of the metabolite. The enzymatic conjugations are carried out by a family of isozymes referred to as the glutathione transferases (Boyland and Chasseaud, 1969, Jakoby, 1978). These enzymes constitute up to 10% and 3% of the extractable protein from rat (Jakoby et al., 1976) or human liver (Kamisaka et al., 1975), respectively. The isozymes have a wide range of overlapping

Table 3. Proposed Functions of Glutathione.

Function	[Reference]
Leukotriene Biosynthesis	
Leukotriene-A ₄ + GSH ^a ---> Leukotriene-C ₄	[1]
Peroxide Metabolism	
H ₂ O ₂ + 2GSH ---> 2H ₂ O + GSSG ^b	[2]
ROOH ^c + 2GSH ----> ROH ^d + H ₂ O + GSSG	[3]
Free Radical Scavenger	
HO· + GSH ---> H ₂ O + GS·	[4]
Storage and Transport Form for Cysteine	[5]
Amino Acid Transport Across Cellular Membranes	[6]
Xenobiotic Metabolism	
X ^e + GSH ----> GSX	[7]

^aGSH = Glutathione, ^bROOH = Organic hydroperoxide,

^cGSSG = Glutathione disulfide, ^dROH = Organic alcohol

^eX = Xenobiotic

[1] = Hammarstrom et al. (1985), [2] = Cohen and Hochstein (1963),

[3] = Flohe and Gunzler (1974), [4] = Bump and Brown (1990), [5] = Tateishi and Sakamoto (1983), [6] = Meister (1978), [7] = Chasseaud (1979)

substrate specificity and exhibit tissue specific isozyme expression (Ketterer, 1986, Mannervik, 1985). Substrates for the glutathione transferases are characteristically hydrophobic and possess one or more electrophilic centers.

Glutathione conjugates formed in the liver may be excreted into the plasma or bile via specific transport processes for glutathione S-conjugates (Sies, 1988). In either case the initial conjugation is the first step in the formation of mercapturic acids (Tate, 1980). Figure 1 outlines the conversion of glutathione S-conjugates to mercapturic acids. This sequence of enzymatic transformations has become known as the mercapturic acid pathway.

The conjugation of glutathione to the reactive electrophilic center of xenobiotic metabolites generated during their metabolism would seem to serve two purposes, 1) the removal of the chemically reactive electrophile thereby destroying its ability to react with other cellular constituents and 2) aiding in the excretion of the xenobiotic via increased hydrophilicity and targeting it for excretion as a glutathione S-conjugate. Glutathione conjugation has become well recognized as an overall detoxification process.

It is true that the vast majority of glutathione conjugations seem to result in protection of the cell and detoxification of the xenobiotic. However, it is now recognized that glutathione conjugation can result, either directly or indirectly, in the production of a more toxic substance (Anders et al., 1988, Monks et al., 1990). The formation of a more toxic compound, which we refer to as bioactivation, could arise by at least two different mechanisms. The glutathione-xenobiotic conjugate may 1) possess a chemically reactive functional group not present

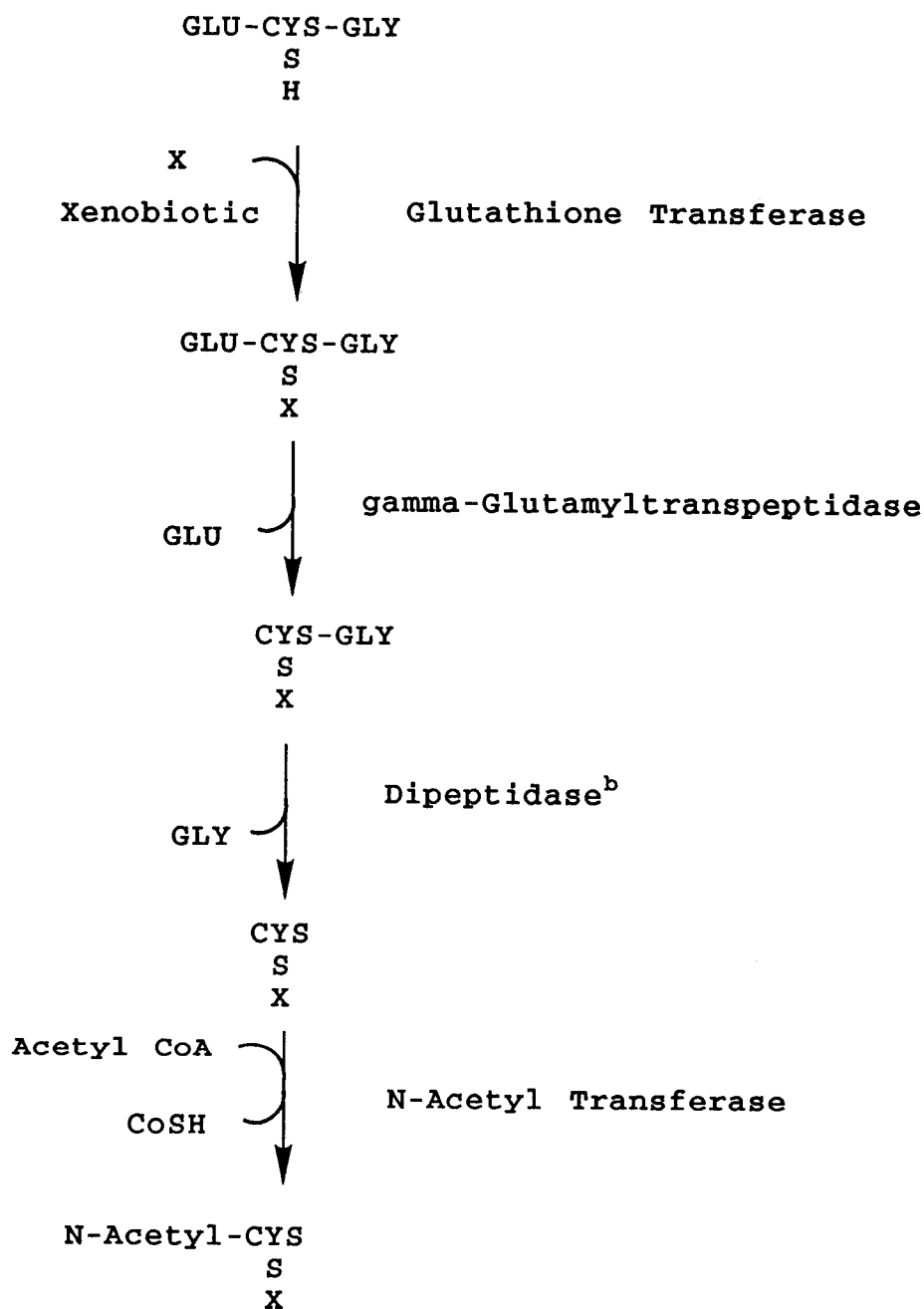


Figure 1. The Mercapturic Acid Pathway^a.

^aTate (1980). ^bA variety of dipeptidases are thought to catalyze this reaction.

in the original xenobiotic, or 2) be a substrate for further metabolism which results in the production of a toxic species. In addition, the conjugation may be reversible and could result in concentrating the xenobiotic in specific tissues which are more susceptible to insult following the release of the xenobiotic from the glutathione S-conjugate.

The focus of my investigations, as delineated in this thesis, concern the role of glutathione and glutathione conjugation reactions in the metabolism and toxicity of 1,2-dihaloethanes and alpha-naphthylisothiocyanate.

1,2-Dihaloethanes: Metabolism and Toxicity

The 1,2-dihaloethanes are a very important class of compounds in that they have served a variety of purposes in our society, such as seed and soil fumigants, gasoline additives, industrial solvents and most importantly as starting material for vinyl halide synthesis which is used primarily in plastics manufacture (Fishbein, 1980). Such extensive utilization has led to an increasing concern for the potential adverse effects that these compounds may have on exposed individuals and our environment. Unfortunately, the 1,2-dihaloethanes have become environmental contaminants that can be found in the air and water supplies in the United States and around the world.

1,2-Dichloroethane and 1,2-dibromoethane are produced and used in the greatest amounts and therefore are the predominate 1,2-dihaloethanes of concern. Both have proved to be toxic to humans and laboratory animals (WHO, 1987, IARC, 1977). Acute lethal oral doses of these 1,2-dihaloethanes characteristically result in central nervous system depression, fatty degeneration and centrilobular necrosis in the liver and proximal tubule necrosis in the kidneys of exposed humans and laboratory animals.

1,2-Dichloro- and 1,2-dibromoethane have also been shown to be mutagenic in a variety of animal and bacterial bioassays (Fabricant and Chalmers, 1980, Hooper *et al.*, 1980) and carcinogenic in mice and rats (National Cancer Institute, 1978, Weisburger, 1977). Based upon the available data 1,2-dichloro- and 1,2-dibromoethane are considered to be potential human carcinogens.

Metabolism of the 1,2-dihaloethanes to chemically reactive species is thought to be necessary for the

development of 1,2-dihaloethane toxicity, mutagenicity and carcinogenicity. Two principal pathways have been proposed for 1,2-dihaloethane metabolism, mixed-function oxidation and glutathione transferase catalyzed conjugation with glutathione (van Bladeren *et al.*, 1981, Guengerich *et al.*, 1980). These two pathways appear to be in direct competition with one another and each can yield reactive metabolites as outlined in figure 2.

The mixed-function oxidation of 1,2-dihaloethanes would yield the respective 2-haloacetaldehyde and possibly lead to the formation of 2-haloethanol or 2-haloacetic acid via alcohol dehydrogenase and aldehyde dehydrogenase, respectively (Shih and Hill, 1981, Guengerich *et al.*, 1980). 2-Haloacetaldehydes are known to be good alkylating agents and may contribute to the toxicity and covalent binding of 1,2-dihaloethanes to cellular macromolecules.

Direct conjugation of glutathione with 1,2-dihaloethane is catalyzed by the glutathione transferases, a family of isozymes with overlapping substrate specificity. The glutathione transferases have been classified into three classes, alpha, mu and pi (Mannervik *et al.*, 1987). Each enzyme consists of two subunits from within the same class (Table 4).

Investigations with purified glutathione transferases have shown that 1,2-dichloro- and 1,2-dibromoethane are substrates for most of the isozymes (Cmarik *et al.*, 1990, Rannug *et al.*, 1978). The product of this conjugation is a glutathione sulfur half-mustard (Edwards *et al.*, 1970, Rannug *et al.*, 1978). The sulfur half-mustard functionality may undergo an internal cyclization characteristic of such a functional group. Displacement of the beta halogen by the sulfur produces a cyclic three-membered ring referred to as an episulfonium ion (Smit *et al.*, 1978). This three-

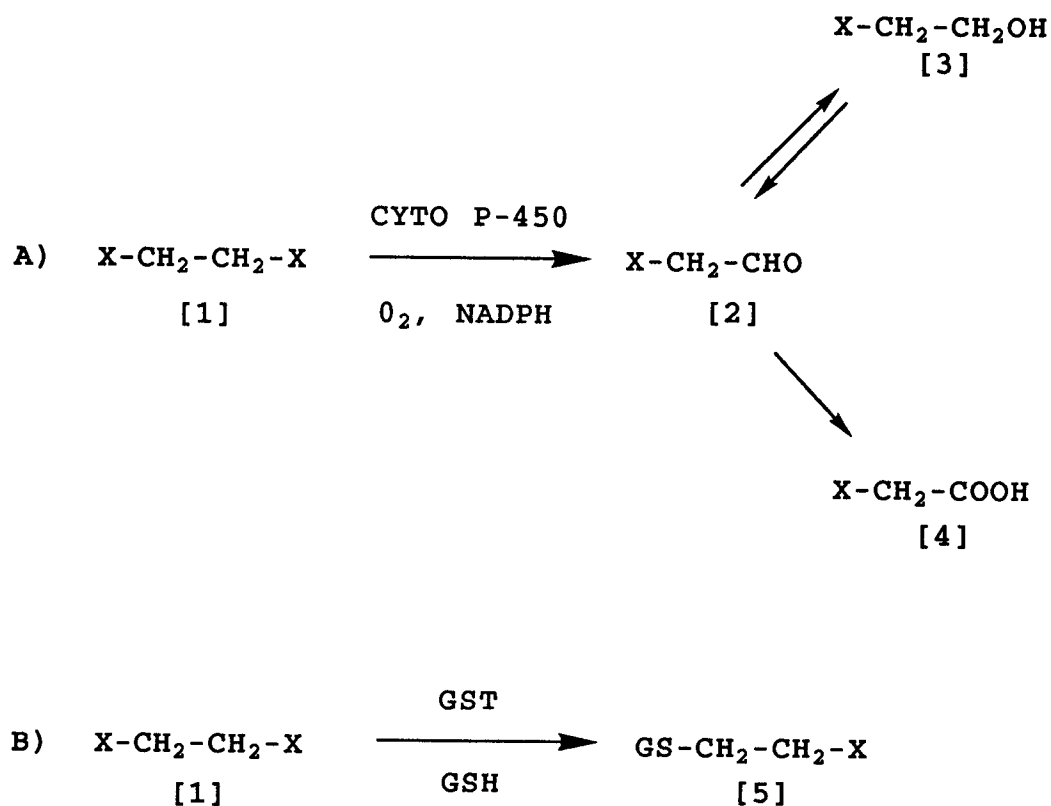


Figure 2. Principle Routes of 1,2-Dihaloethane Metabolism^a. The metabolic scheme for mixed-function oxidase (A) and glutathione transferase (B) metabolism of 1,2-dihaloethanes (X = Br or Cl). 1,2-Dihaloethane, [1]; 2-haloacetaldehyde, [2]; 2-haloethanol, [3]; 2-haloacetic acid, [4]; S-(2-haloethyl)glutathione, [5]; glutathione transferase, (GST), and glutathione (GSH). ^aAdapted from Anders and Livesey (1980) and Guengerich et al. (1980).

Table 4. Mammalian Glutathione Transferases^a.

Species	Cytosolic			<u>Microsomal</u>
	Alpha	Mu	Pi	
Rat	1-1	3-3	7-7	present
	1-2	3-4		
	2-2	4-4		
	8-8	6-6		
		3-?		
Mouse	4-4	1-1	3-3	
		2-2		
Homo	$\alpha, \beta, \chi, \delta, \epsilon$	μ	π	present

Individual subunits are denoted by numbers. Greek letters denote specific enzymes. ^aMannervik et al., (1987)

membered ring is a good electrophile due to the strained nature of the ring and can be a potent alkylating agent toward protein and nucleic acids as demonstrated for other sulfur mustards (Brookes and Lawley, 1961, Hartwell, 1946).

The recognition that two distinct metabolic pathways exist for 1,2-dihaloethane metabolism and that each produces reactive metabolites has raised questions concerning the contribution of each metabolic route in 1,2-dihaloethane toxicity, mutagenicity and carcinogenicity.

The mutagenicity of 1,2-dibromoethane and 1,2-dichloroethane in bacterial mutagenicity assays was enhanced by the addition of the S9 supernatant fraction of liver homogenate (Rannug et al., 1978). Further investigations demonstrated that the enhanced mutagenicity was dependent upon glutathione and the 100,000g supernatant fraction

(Rannug *et al.*, 1978, van Bladeren *et al.*, 1980). Rannug *et al.* (1978) also demonstrated that purified preparations of glutathione transferase and glutathione enhanced the mutagenicity of these 1,2-dihaloethanes. S-(2-Chloroethyl)-glutathione, the putative metabolite of glutathione transferase activation of 1,2-dichloroethane, and analogs of this glutathione S-conjugate have been shown to be mutagenic (Humphreys *et al.*, 1990). Altogether, these results demonstrate the importance of glutathione conjugation in the mutagenicity of these 1,2-dihaloethanes.

1,2-Dihaloethane carcinogenicity is also thought to be directly related to metabolism via glutathione transferase bioactivation. Various observations have given support to this contention. This would include the identification of the major DNA adduct in rat liver and kidney following 1,2-dibromoethane exposure as S-(2-(N⁷-guanyl)ethyl)glutathione (Koga *et al.*, 1986, Inskeep *et al.*, 1986). In addition, disulfiram, a drug that reduces liver mixed-function oxidase metabolism and increases glutathione transferase activity was found to greatly enhance the carcinogenic potential of 1,2-dibromoethane and 1,2-dichloroethane (Plotnik *et al.*, 1980, Cheever *et al.*, 1990). These data along with those concerning mutagenicity provide support for the proposed role of glutathione transferase metabolism in 1,2-dihaloethane carcinogenicity.

The role of either metabolic pathway in the acute toxicity of 1,2-dihaloethanes is uncertain. The liver and kidney represent the major target organs for toxicity and both possess mixed-function oxidase and glutathione transferase activities. Pretreatment of animals with mediators of these two pathways have been shown to modulate 1,2-dihaloethane hepatotoxicity. The use of deuterated 1,2-dibromoethane was shown to elevate serum glutamic-pyruvic

transaminase levels in mice as compared to non-deuterated 1,2-dibromoethane (White *et al.*, 1983). Reduced mixed-function oxidase metabolism and increased glutathione transferase metabolism was thought responsible for the increased toxicity. Diethyldithiocarbamate, an inhibitor of mixed-function oxidase activity, potentiates 1,2-dibromoethane toxicity (Nachtomí, 1980). Disulfiram was also shown to potentiate 1,2-dibromoethane toxicity (Wong *et al.*, 1982). Altogether, these data suggest that glutathione transferase metabolism promotes 1,2-dibromoethane toxicity. However, these observations in no way exclude the potential contribution of mixed-function oxidase metabolism in 1,2-dihaloethane toxicity. The role of either metabolic pathway in 1,2-dihaloethane toxicity remains to be unequivocally defined.

There is much interest in defining the metabolism and mechanism of action of the 1,2-dihaloethanes. This is primarily due to the continued use of these compounds in the world today and recognition that they are becoming common environmental pollutants.

***alpha-Naphthylisothiocyanate: Hepatic Injury and
Cholestasis***

Cholestasis, the reduction or cessation of bile flow, may develop in humans as a side effect of a variety of drugs or in association with viral infections. For obvious reasons the investigation of drug-induced cholestasis has required the development of practical animal models that mimic the drug-induced cholestasis observed in humans. However, the development of cholestasis in humans as well as in animal models is not well understood. This stems from the lack of a complete understanding of the complexities involved in bile formation and bile flow. Table 5 lists potential sites for drug action that could lead to the development of cholestasis.

One of the most widely used animal models employs alpha-naphthylisothiocyanate as the cholestatic agent. alpha-Naphthylisothiocyanate produces intrahepatic cholestasis in susceptible species within 24 hours after acute administration (Plaa and Priestly, 1977). The development of cholestasis is associated with hyperbilirubinemia, elevation of serum total bile acid content, and increased serum levels of marker enzymes of liver injury (El-Hawari and Plaa, 1979). The principle lesions of acute administration of alpha-naphthylisothiocyanate in the liver appear to involve degeneration and necrosis of the interlobular bile ducts and focal necrosis of hepatocytes in the portal region (Connolly et al., 1988, Goldfarb et al., 1962).

The mechanism of alpha-naphthylisothiocyanate-induced cholestasis is unknown. Investigations with modulators of microsome mixed-function oxidase activity have suggested

Table 5. Potential Sites of Action in Drug-induced Cholestasis.

Hepatocyte
Synthesis of bile acids
Transport of bile acids across canalicular membrane
Ion transport across canalicular membrane
Tight junctional permeability
Hepatocyte viability
Bile Duct
Bile duct epithelium secretions
Bile duct permeability
Bile duct epithelium viability

that metabolism of alpha-naphthylisothiocyanate to a toxic metabolite is responsible for its toxic effects. Phenobarbital and 3-methylcholanthrene pretreatment increased and SKF 525-A, piperonyl butoxide and disulfiram decreased alpha-naphthylisothiocyanate-induced hyperbilirubinemia, an indirect measure of cholestasis (El-Hawari and Plaa, 1977). However, a reactive metabolite has yet to be identified and its mode of action determined. It is also possible that the inhibitors and inducers of mixed-function oxidase metabolism may modulate other intracellular systems that interact with alpha-naphthylisothiocyanate to potentiate or inhibit its toxic effects. The modulation of glutathione and glutathione transferase activity have been proposed as one possibility (Dahm and Roth, 1991).

The effect of hepatic glutathione modulation on alpha-naphthylisothiocyanate toxicity was investigated (Dahm and Roth, 1991). The authors reported that depletion of hepatic

glutathione gave rise to an inhibition of cholestasis and hepatic toxicity. Further, that cholestasis developed in concert with the repletion of tissue glutathione. These data strongly implicate glutathione in alpha-naphthylisothiocyanate-induced cholestasis and liver injury. Note that glutathione appears to function not in protection but in potentiating alpha-naphthylisothiocyanate toxicity.

Interest in chemically-induced toxicities involving glutathione in a role of activation has resulted in the investigation of alpha-naphthylisothiocyanate toxicity. In this regard I have investigated the effect of alpha-naphthylisothiocyanate on intracellular glutathione and toxicity in freshly isolated rat hepatocytes. The direct interaction of alpha-naphthylisothiocyanate with glutathione was also studied. The results of my investigations are presented in chapter 5 of this thesis.

GLUTATHIONE SULFUR HALF-MUSTARDS AS ALKYLATING AGENTS¹

Introduction

As with many chemical toxicants, exposure of laboratory animals to 1,2-dihaloethanes results in the covalent binding of 1,2-dihaloethane to tissue protein, RNA and DNA (Arfellini *et al.*, 1984, Hill *et al.*, 1978, Igwe *et al.*, 1986, Reitz *et al.*, 1982). The covalent binding of 1,2-dibromoethane and 1,2-dichloroethane to protein and DNA has been shown to be dependent upon metabolic activation (Guengerich *et al.*, 1980, Shih and Hill, 1981, Banerjee and Van Duuren, 1979). The use of subcellular fractions *in vitro*, enzyme inhibitors *in vivo*, and identification of the major 1,2-dibromoethane-derived DNA adduct as a glutathione conjugate have led to the generally accepted conclusion that DNA alkylation results principally from the formation of glutathione sulfur half-mustards. However, the covalent binding 1,2-dihaloethane to proteins is not well defined even though it accounts for the majority of the total 1,2-dihaloethane covalently bound to cellular macromolecules (Hill *et al.*, 1978, Arfellini *et al.*, 1984).

The level of 1,2-dibromoethane covalently bound to rat liver proteins, as reported by Igwe *et al.* (1986), suggest that up to 7% of the total protein of the liver may have undergone alkylation (assuming one alkylation event per 50,000 dalton protein). However, very little is known about

¹Much of this thesis chapter has been published in **Chemical Research in Toxicology**, 1989, Vol 2(6), 455-460. Figures and Tables have been reproduced with permission from the American Chemical Society.

the nature of the alkylating agent(s) and the specific amino acid residues undergoing alkylation.

There are two principle metabolic routes for the activation of 1,2-dihaloethanes, both produce reactive metabolites that could contribute to the observed covalent binding. 2-Haloacetaldehydes, the product of 1,2-dihaloethane mixed-function oxidation, are known alkylating agents toward protein and nucleic acids (Banerjee et al., 1979, Kusmieriek and Singer, 1982). In contrast, little is known about the ability of the glutathione sulfur half-mustards, the putative 1,2-dihaloethane metabolites of the glutathione transferase pathway, to alkylate proteins. However, it is well established that sulfur mustards can be active alkylating agents toward protein and DNA. Thus it is very likely that the glutathione sulfur half-mustards are also active alkylating agents toward both protein, RNA and DNA.

1,2-Dihaloethane exposure may result in the *in vivo* formation of glutathione sulfur half-mustards. 1,2-Dibromoethane and 1,2-dichloroethane are substrates for the glutathione transferases and the major DNA adduct identified in 1,2-dibromoethane treated rats is apparently the result of intracellular formation of S-(2-bromoethyl)glutathione. In addition, S-(2-chloroethyl)glutathione was recently identified in the bile of 1-bromo-2-chloroethane treated rats (Marchand and Reed, 1989). This was the first direct evidence demonstrating the *in vivo* formation of glutathione sulfur half-mustards.

Our laboratory has synthesized S-(2-chloroethyl)-glutathione and S-(2-chloroethyl)-L-cysteine, the putative glutathione sulfur half-mustard and cysteinyl analog thought to arise from the conjugation of glutathione with 1,2-dichloroethane. Investigations with these compounds have

shown that they can act as alkylating agents toward the model nucleophile 4-(p-nitrobenzyl)pyridine (Reed and Foureman, 1986) as well as with 2'-deoxyguanosine to yield S-(2-(N⁷-guanyl)ethyl)glutathione adduct, the same DNA adduct found *in vivo* following 1,2-dibromoethane exposure (Foureman and Reed, 1987). In addition, these sulfur half-mustards were found to be potent nephrotoxins when administered to rats (Elfarra *et al.*, 1985, Kramer *et al.*, 1987).

There are a great many questions that have yet to be answered concerning 1,2-dihaloethane toxicity; such as the importance of covalent binding in 1,2-dihaloethane toxicity and the role of each of the metabolic pathways in contributing to that covalent binding. The ability to prepare S-(2-chloroethyl)glutathione and its cysteinyl analog and the lack of information concerning protein alkylation by specific glutathione sulfur half-mustards have prompted me to investigate further the ability of glutathione sulfur half-mustards to function as alkylating agents. Specifically, I have characterized the alkylation of a variety of dipeptides, nucleosides and glutathione by S-(2-chloroethyl)-glutathione and S-(2-chloroethyl)-L-cysteine. My observations and a discussion of their importance are presented in this chapter.

Materials and Methods

Chemicals:

L-Cystinyl-bis-L-tyrosine, L-glycyl-L-tyrosine and L-lysyl-L-tyrosine were obtained from Chemical Dynamics Corp. (South Plainfield, NJ). The other dipeptides, amino acids, nucleosides and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Dibromoethane, 1-bromo-2-chloroethane, and iodo[2-¹⁴C]acetic acid (56 mCi/ mmol) were purchased from Eastman Kodak Co. (Rochester, NY), Aldrich Chemical Co. (Milwaukee, WI), and Amersham (Arlington Heights, IL), respectively. All of the chemicals used were reagent grade or better.

Preparation of S-(2-chloroethyl)glutathione and S-(2-chloroethyl)cysteine:

S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine were synthesized as reported by Reed and Foureman (1986). Preparative high-performance liquid chromatography (HPLC) isolation routinely yielded S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine with purities of >95% and ≥90%, respectively. The impurities corresponding to the S-(2-hydroxyethyl)- hydrolysis products as determined by analytical HPLC.

Extent of Dipeptide and Nucleoside Alkylation:

The extent of dipeptide and nucleoside alkylation was investigated for reactions of 1.0 mM dipeptide or nucleoside in 0.15 M KH₂PO₄ buffer (pH = 7.4) with 14 mM S-(2-chloroethyl)glutathione or S-(2-chloroethyl)-L-cysteine added as the dry powder. Incubation for 6 hours at 37°C was followed by analytical HPLC analysis of the reaction mixtures

employing mobile phases consisting of 5.0 mM KH_2PO_4 and methanol (1.0 to 60%), pH = 4.6. One millimolar cysteinyltyrosine was prepared by reduction of 0.5 mM cystinyl-bis-tyrosine with dithiothreitol (DTT) for approximately 40 minutes followed by ethylacetate extraction to remove unreacted DTT. Reduction to cysteinyltyrosine was shown to be essentially complete by HPLC analysis. Dipeptide adducts were detected by their ultraviolet absorbance (UV) at 274 nm. Depending upon the parent compound's maximum absorption, UV absorbances for the nucleoside adducts ranged from 250 to 280 nm.

Reaction Rate Determination:

Reaction rates were determined with reaction mixtures containing 4.0 mM dipeptide or nucleoside with 14 mM S-(2-chloroethyl)glutathione or S-(2-chloroethyl)-L-cysteine in the same manner described above. At various times aliquots of these reaction mixtures were mixed with 100 fold excess β -mercaptoethanol (compared to conjugate) to stop the reaction. These β -mercaptoethanol treated fractions were frozen with liquid nitrogen, lyophilized, and redissolved to one-fourth the original volume with a solution containing an internal standard. 7-Methylguanine, valyltyrosine, and glycytyrosine were used as internal standards for the reaction mixtures of 2'-deoxyguanosine, cysteinyltyrosine, and histidyltyrosine, respectively. Inclusion of an internal standard corrected for the day to day variability in detector sensitivity and provided a convenient method for determination of the amounts of adduct formed. Histidyltyrosine values were corrected to correspond to that relative to valyltyrosine to allow direct comparison with the cysteinyltyrosine data.

Determination of the Extent and Rate of Glutathione Alkylation:

Determination of the extent and rate of alkylation of glutathione were performed as described above except that reaction mixtures were treated with 1-fluoro-2,4-dinitrobenzene in the presence of excess sodium bicarbonate for 18-24 hours and aliquots subjected to HPLC analysis as described by Fariss and Reed (1987), utilizing gamma-glutamylglutamate as an internal standard.

Bulk Preparation and Isolation of Adducts:

Large-scale production of cysteinyltyrosine and histidyltyrosine adducts were carried out with essentially the same conditions as described above. Isolation and desalting of the S-(2-chloroethyl)glutathione adducts of cysteinyltyrosine and histidyltyrosine as well as the S-(2-chloroethyl)-L-cysteine adduct of cysteinyltyrosine were done with the preparative HPLC column and a methanol/water mobile phase. Lyophilization was performed to remove the mobile phase. The S-(2-chloroethyl)-L-cysteine adduct of histidyltyrosine was separated from parent material on the analytical column with a phosphate buffer/methanol mobile phase and desalted as described. The glutathione adduct of S-(2-chloroethyl)glutathione was prepared by mixing two equivalents of glutathione with one equivalent of 1,2-dibromoethane in 30% ethanol in the presence of triethanolamine, pH = 9.0, for 24 hours at room temperature (Nachtomi, 1970). Bulk preparation of the S-(2-chloroethyl)-L-cysteine adduct of glutathione was accomplished by reacting S-(2-chloroethyl)-L-cysteine with two equivalents of glutathione in 0.15 M KH_2PO_4 at 37°C for 12 hours. Preparative isolation of the glutathione adducts was performed utilizing the preparative column described

earlier and mobile phases consisting of (4-8%) methanol, 0.1% acetic acid in water. Purity of these isolated adducts, as judged by HPLC, was $\geq 90\%$.

Adduct Analysis:

The ultraviolet spectral characteristics of the adducts were determined for 1.0 mM solutions of purified adducts in water at pH 6.0 and 12.0. Negative ion fast atom bombardment mass spectrometry (FAB-MS) was performed by dissolving adducts in a matrix of thioglycerol:glycerol (2:1) and ammonium hydroxide on the probe. Proton noise decoupled ^{13}C nuclear magnetic resonance spectroscopy (NMR) was determined after dissolving the isolated adducts in deuterium oxide.

Chemical Characterization of the Cysteinylytyrosine Adducts:

Iodoacetic acid binding studies were performed by first treating the S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine adduct of cysteinylytyrosine with DTT (4 times excess) for 40 min at 37°C followed by extraction with ethylacetate. Aliquots (100 μl) of the aqueous solution were added to 50 μl of a 28 mM solution of radiolabeled iodoacetic acid (0.37 microcuries/mmol) and incubated at room temperature at pH = 9.0 for 1 hour in the dark. Aliquots were analyzed by analytical HPLC, the eluate was collected in 1-mL fractions and 0.3 ml of each fraction were dissolved in 4.0 ml of formula-963 scintillation cocktail for assay of radioactivity.

Instrumentation:

Analysis of the dipeptide and nucleoside adducts was performed with either a LKB 2152 controller, two 2150 LC Pumps and a Spectroflow 757 UV detector or a Kontron

Programmer series 200, two no. 414 LC pumps and a UVikon 730 LC detector. A Hewlett Packard 3388A integrator, equipped with two chart recorders, recorded and integrated the signals from the detectors. Spherosorb ODSII reverse phase columns (4.6 x 250mm, 5 micron, Custom LC, Inc., Houston, TX) were used for the analytical applications whereas a Partisil ODS2 reverse phase column (10 x 500mm, 10 micron, Alltech, Deerfield, IL) was utilized for large-scale isolations. Mobile phases were prepared from HPLC-grade solvents and/or Milli-Q water and filtered through 0.45 micron nylon filters before use.

NMR analysis was performed with either a Bruker AM400 or AM300 NMR spectrometer operating at 400 and 300 MHz respectively. FAB-MS (negative mode) was performed with a Kratos MS50 mass spectrometer. Ultraviolet spectral characterization of the dipeptide adducts was performed utilizing a Hewlett-Packard 8452A Diode array spectrophotometer. Ultraviolet spectra of 2'-deoxyguanosine adducts were recorded with a LKB 2140 rapid spectral detector as they eluted from the HPLC column. Radioactivity was measured with a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 2450.

Results

The first phase of this investigation was concerned with the determination of the extent of alkylation of various dipeptides, nucleosides and glutathione by S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine. The aim was to determine which of the compounds undergo detectable levels of alkylation and of these, which would be best suited for further characterization and comparison. To accomplish this I prepared 1.0 mM solutions of dipeptide, nucleoside and glutathione in phosphate buffer and added enough S-(2-chloroethyl)glutathione or S-(2-chloroethyl)-L-cysteine as the dry powder to yield 14 mM sulfur half-mustard. The mixtures were incubated for 6 hours at 37°C and the amount of adduct present after incubation was determined.

As is shown in table 6 all of the dipeptides gave rise to some level of alkylation. The extent of alkylation under these conditions exhibited the following pattern; cysteinyl-tyrosine alkylation was >> than histidyltyrosine which was > the other remaining dipeptides for both S-(2-chloroethyl)-glutathione and S-(2-chloroethyl)-L-cysteine. Significant differences between S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine dipeptide alkylation were observed with lysyltyrosine, glycytyrosine and histidyltyrosine. S-(2-chloroethyl)glutathione alkylation of glycytyrosine gave rise to low but detectable levels of adduct whereas S-(2-chloroethyl)-L-cysteine did not. However, this difference is most likely the result of the detection limit and therefore is not likely to be of significance.

Alkylation of glutathione by both S-(2-chloroethyl)-glutathione and S-(2-chloroethyl)-L-cysteine was extensive

Table 6. Dipeptide and Glutathione Alkylation by S-(2-Chloroethyl)glutathione (CEG) and S-(2-Chloroethyl)-L-cysteine (CEC)^a.

Compound	% Alkylated ^b	
	CEG	CEC
Glycyltyrosine	1.13 ± 0.16	<LD ^c
Glycyltryptophan ^{**}	1.74 ± 0.12	2.77 ± 0.36
Lysyltyrosine [*]	2.93 ± 0.15	4.27 ± 0.68
Histidyltyrosine ^{**}	6.86 ± 0.16	8.85 ± 0.23
Cysteinyltyrosine	87.80 ± 4.80 ^d	94.50 ± 7.22 ^d
Glutathione	93.00 ± 2.30	91.60 ± 3.90

^aDipeptides or glutathione were incubated with CEG or CEC (1:14 ratio of dipeptide:conjugate) for 6 hours at 37°C and then analyzed by reverse-phase HPLC. Table entries are the average of at least three separate reaction mixtures ± the standard error. Student's paired t-test was used to evaluate the differences in CEG vs CEC alkylations for statistical significance. ^b(Product Peak Area/Total Dipeptide Derived Area)×100%. ^c<LD = Below Detection Limit (< 1 nmol / mL reaction). ^dDetermined as the percentage of cysteinyltyrosine present at the start of reaction.

^{*}CEG alkylation is statistically different from CEC alkylation (P<0.05). ^{**}CEG alkylation is statistically different from CEC alkylation (P<0.01).

(approximately 90% alkylated). No significant difference was observed for the extent of alkylation by S-(2-chloroethyl)glutathione in comparison to S-(2-chloroethyl)-L-cysteine.

Nucleoside alkylation was not nearly as extensive as that for the dipeptides (Table 7). The only nucleoside to yield detectable levels of alkylation was 2'-deoxyguanosine. 2'-Deoxyguanosine alkylation was very low (less than 2.5% alkylated). S-(2-Chloroethyl)glutathione gave rise to a small but significantly greater amount of adduct than S-(2-chloroethyl)-L-cysteine.

To further the characterization of these sulfur half-mustards, the initial rates of reaction were determined for S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine with cysteinyltyrosine, glutathione, histidyltyrosine and 2'-deoxyguanosine. The reaction mixtures consisted of 4.0 mM compound and 14 mM sulfur half-mustard in a phosphate buffer. The reaction mixtures were incubated at 37°C and aliquots were taken and assayed. The results are given in table 8.

The alkylation rates of cysteinyltyrosine and glutathione were markedly greater than that for any of the other compounds tested. Cysteinyltyrosine alkylation occurred at rates 10-fold greater than histidyltyrosine and 50 times greater than 2'-deoxyguanosine for both S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine. The rate of glutathione alkylation by S-(2-chloroethyl)-glutathione was greater than that for cysteinyltyrosine whereas S-(2-chloroethyl)-L-cysteine alkylation of glutathione was less than that of cysteinyl-tyrosine. The rates of glutathione, histidyltyrosine and 2'-deoxyguanosine alkylation by S-(2-chloroethyl)glutathione was significantly

Table 7. Nucleoside Alkylation by S-(2-Chloroethyl)-glutathione (CEG) and S-(2-Chloroethyl)-L-cysteine (CEC)^a.

Nucleoside	% Alkylated ^b	
	CEG	CEC
2'-Deoxyguanosine**	2.19 ± 0.26	1.45 ± 0.11
2'-Deoxyadenosine	ND ^c	ND
2'-Deoxycytidine	ND	ND
Thymidine	ND	ND

^aNucleosides were incubated with CEG and CEC (1:14 ratio nucleoside:conjugate) for 6 hours at 37°C (pH = 7.4) followed by reverse-phase HPLC analysis of the mixtures. Table entries are the average of at least three separate reaction mixtures ± the standard error. Student's paired t-test was used to evaluate the statistical significance of the differences between CEG vs CEC alkylations.

^b(Product Peak Area/Total Nucleoside Derived Area)×100%.

^cLD = Below Detection Limit (< 1 nmol/mL reaction)

**CEG alkylation is statistically different from CEC alkylation (P<0.01).

Table 8. Initial Reaction Rates^a.

Compound	Conjugate	
	CEG ^b	CEC ^b
Glutathione ^{**}	325.5 ± 13.1	209.6 ± 26.5
Cysteinyltyrosine	255.0 ± 20.4	268.9 ± 10.7
Histidyltyrosine ^{**}	16.3 ± 2.8	26.5 ± 2.3
2'-Deoxyguanosine ^{**}	4.9 ± 0.4	3.7 ± 0.3

^aReaction rates were determined within the first 3 minutes of mixing 4.0 mM compound with 14.0 mM S-(2-chloroethyl)glutathione (CEG) or S-(2-chloroethyl)-L-cysteine (CEC) at 37°C (pH = 7.4). Reactions were stopped with the addition of beta-mercaptoethanol. HPLC separation allowed quantification of the amount of adduct formed. Student's paired t-test was used to evaluate the statistical significance between CEG and CEC alkylation rates.

^{**}The rate of CEG alkylation was statistically different (P<0.01) from CEC. ^bnmol product formed per minute per mL ± standard error (n ≥ 3).

different than that observed for S-(2-chloroethyl)-L-cysteine.

A variety of chemical and physical techniques were utilized to determine the sites of alkylation of glutathione, cysteinyltyrosine, histidyltyrosine and 2'-deoxyguanosine.

The data concerning 2'-deoxyguanosine alkylation suggest that alkylation occurred at the N⁷ position yielding the respective S-(2-(N⁷-guanyl)ethyl)-adduct as reported by Foureman and Reed (1987). This determination was based upon HPLC retention times and UV spectra comparisons.

The isolated adducts of cysteinyltyrosine, histidyltyrosine and glutathione were subjected to FAB-MS analysis to determine the relative molecular weights of each adduct. In this way we could assess the degree to which the dipeptides and glutathione were alkylated, ie. the addition of one or more sulfur half-mustard moieties. The molecular ions observed suggest that almost all of the adducts are the result of a single addition of sulfur half-mustard. The only exception to this was found for the alkylation of cysteinyltyrosine by S-(2-chloroethyl)-L-cysteine as shown in figure 3. Here the major molecular ion peak at 430 atomic mass units represents the addition of a single S-(2-chloroethyl)-L-cysteine moiety. The molecular ion peak at 577 atomic mass units would represent the addition of two S-(2-chloroethyl)-L-cysteine moieties. The nature of this technique does not allow the determination the amounts of mono- verses dialkylated adducts from the observed peak intensities and so it is uncertain just what proportion of the cysteinyltyrosine adduct is mono- verses dialkylated. However, the data suggest that the amount of dialkylated cysteinyltyrosine is small because of the limited amount of adducts formed with other dipeptides such as glycyltyrosine.

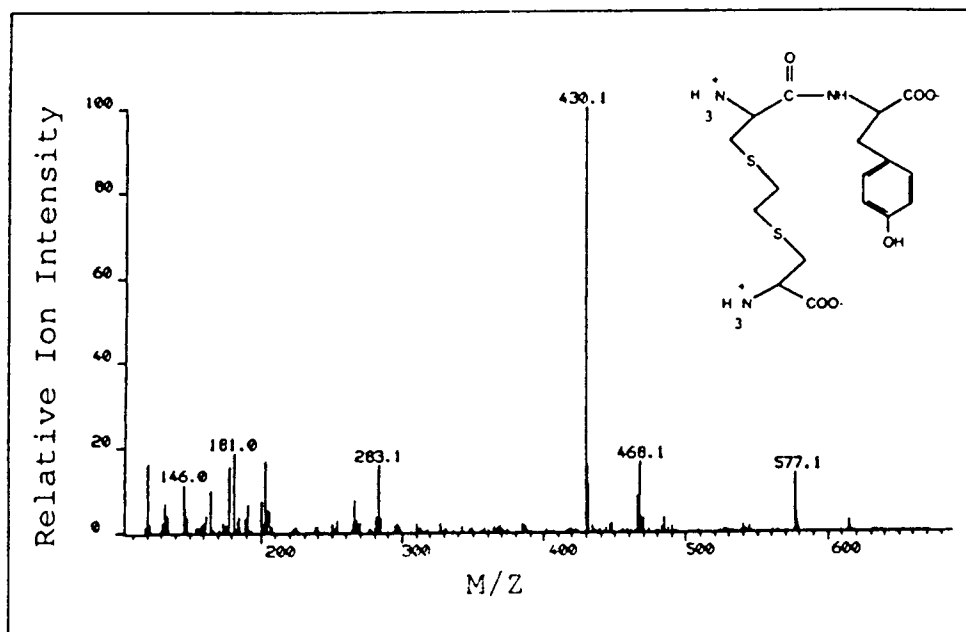


Figure 3. FAB-MS (negative ion) of the S-(2-Chloroethyl)-L-cysteine (CEC) Adduct of Cysteinyltyrosine. Molecular ion peaks at 430 and 577 M/Z correspond to that predicted for the CEC monoalkylated (as shown) and dialkylated cysteinyltyrosine. FAB-MS was performed as described in the "Materials and Methods" section.

The alpha-amino and tyrosyl hydroxyl groups would be the only groups available for alkylation following cysteinyl thiol alkylation and they have proved to not be very reactive.

The alkylation of tyrosine residues was investigated spectroscopically at neutral and alkaline pH's. The spectra obtained are consistent with the notion that the tyrosyl hydroxyl was not a site of alkylation by the sulfur half-mustards. In addition, ^{13}C -NMR analysis of the adducts showed no significant changes in the signals attributed to tyrosyl carbons which would further suggest the lack of any tyrosine alkylation events (Table 9). Acid hydrolysis of cysteinyltyrosine and histidyltyrosine adducts followed by HPLC analysis resulted in the release of free tyrosine from the adducts as shown for the S-(2-chloroethyl)-L-cysteine adduct of cysteinyltyrosine in figure 4.

The alkylation of the imidazole ring of histidyl residues was investigated with ^{13}C -NMR just as was done for the tyrosyl residues. However, there were small shifts in the carbon signals assigned to the imidazole ring. These shifts would suggest that the adducts have a different electronic structure than the parent histidyltyrosine. Thus suggesting that the imidazole ring nitrogens are most likely the site of alkylation.

The presence of free thiol within the cysteinyltyrosine adducts was investigated with radiolabeled iodoacetic acid. The inability of the adducts to covalently bind iodoacetic acid suggests that the adduct does not contain a free thiol.

Together these data suggest that the major sites for alkylation of these dipeptides and glutathione residues with the cysteinyl thiol and imidazole ring nitrogens and not at the tyrosyl hydroxyl.

Table 9. ¹³C NMR Chemical Shift Data for the Tyrosyl and Imidazole Ring Carbons of Cysteinyltyrosine and Histidyltyrosine Adducts.

Chemical Shift ^a (PPM)					
Carbon	Parent	CTEC ^b	CTEG ^b	HTEC ^c	HTEG ^c
Tyrosyl aromatic					
C ^γ	128.4	128.5	128.4	-----	-----
	129.9	-----	-----	129.9	129.8
C ^{δ1} & ^{δ2}	115.8	115.8	115.8	115.7	115.7
C ^{ε1} & ^{ε2}	130.9	130.9	131.0	130.8	130.8
C ^η	154.7	154.9	154.9	154.4	154.5
Histidyl imidazole					
C ^γ	118.1	-----	-----	117.4	118.1
C ^{δ2}	129.7	-----	-----	129.4	127.6
C ^{ε1}	135.9	-----	-----	134.4	134.4

^aAcetonitrile used as reference. ^bCompounds dissolved in D₂O, pH < 2.0. ^cCompounds dissolved in D₂O, pH = 7.0

HTEC = N^{δ1} or ^{ε2}-[2-(S-cysteinyl)ethyl]histidyltyrosine

HTEG = N^{δ1} or ^{ε2}-[2-(S-cysteinyl)ethyl]histidyltyrosine

CTEC = S-[2-(cysteinyl)ethyl]cysteinyltyrosine

CTEG = S-[2-(glutathionyl)ethyl]cysteinyltyrosine

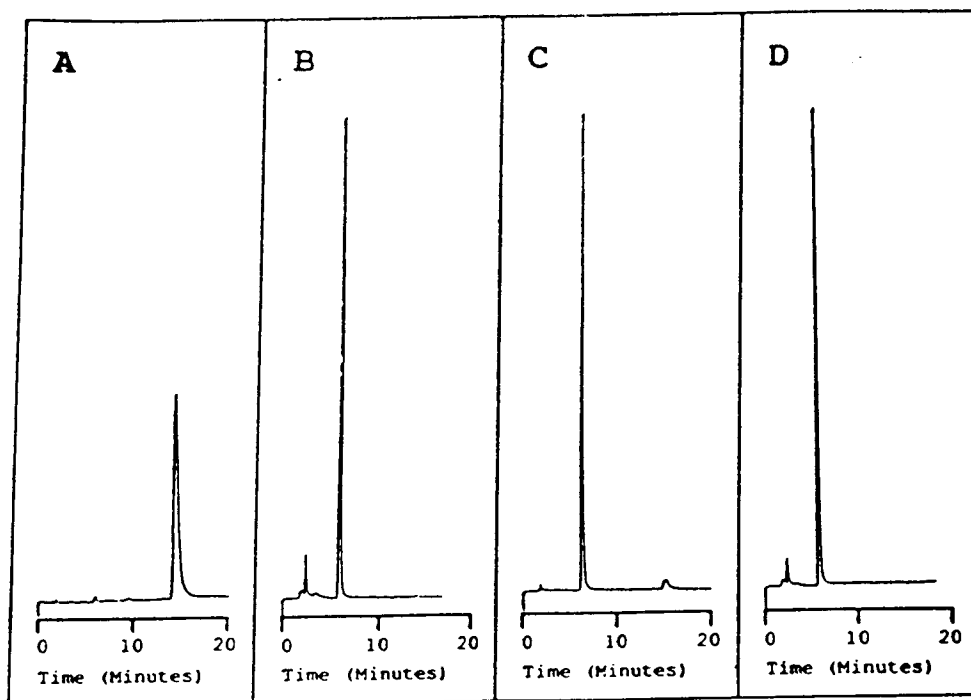


Figure 4. HPLC Analysis of the S-(2-Chloroethyl)-L-cysteine (CEC) Adduct of Cysteinytyrosine (A) Before and After (B) Acid Hydrolysis. Elution of (C) a tyrosine standard and (D) a mixture of tyrosine standard and hydrolysate are given. Chromatograms represent the elution of 274 nm absorbing material from the reverse-phase column as described in the "Materials and Methods" section.

Discussion

This investigation has shown that S-(2-chloroethyl)-glutathione and S-(2-chloroethyl)-L-cysteine, putative metabolites of 1,2-dichloroethane metabolism by glutathione transferases, are direct-acting alkylating agents. A variety of functional groups common to proteins were examined for their susceptibility to alkylation by these sulfur half-mustards. The cysteinyl thiol functional group was shown to be the most active towards alkylation, exhibiting rates of alkylation more than 10-fold greater than the next most reactive functional group. However, each of the dipeptides examined gave rise to some level of alkylation indicating that the sulfur half-mustards can alkylate a variety of functional groups commonly found in proteins.

That 2'-deoxyguanosine alkylation by these sulfur half-mustards was greater than that for any of the other nucleosides examined was not unexpected. Sulfur mustards, in general, are known to react preferentially with the N⁷ position of guanine as compared with potential sites on other nucleoside residues.

What was curious though was the great difference in susceptibility between alkylation of the dipeptides verses the nucleosides. The data suggest that protein alkylation by the glutathione sulfur half-mustard and it's cysteinyl analog could be markedly greater than that for DNA alkylation *in vivo*. However, it must be recognized that the availability and nucleophilicity of specific functional groups within proteins and DNA may vary greatly from those demonstrated with model compounds.

There is no doubt that the conjugation of 1,2-dihalo-

ethanes to glutathione, producing the glutathione sulfur half-mustard, is an activation step. The two sulfur half-mustards examined were very reactive toward cysteinyl thiol, both within the dipeptide cysteinyltyrosine as well as with glutathione. However, this second reaction with glutathione could represent a detoxification reaction *in vivo*. Suggesting that glutathione may also function to detoxify the sulfur half-mustards. The alkylation of glutathione could protect other cellular constituents from alkylation. Protection would depend upon the maintenance of sufficient intracellular levels of glutathione.

Glutathione conjugates formed in the liver are thought to be actively excreted either into the bile or the plasma. In either compartment they may experience further metabolism that could lead to the formation of mercapturic acids, the typical urinary excretion products of glutathione S-conjugates. This transformation from glutathione S-conjugate to mercapturic acid represents the Mercapturic Acid Pathway and results in the production of a variety of intermediates (Figure 1). S-(2-Chloroethyl)-L-cysteine is one of several possible intermediates that could arise during the transformation of S-(2-chloroethyl)glutathione to a mercapturic acid. Each of the intermediates of this pathway possess the sulfur half-mustard functionality and may therefore be an active alkylating agent that contributes to the overall toxicity of the 1,2-dihaloethanes. I have included S-(2-chloroethyl)-L-cysteine in this study to compare its activity as an alkylating agent to that of S-(2-chloroethyl)glutathione. This characterization of S-(2-chloroethyl)-L-cysteine may aid in understanding the importance of glutathione sulfur half-mustard derivatives in 1,2-dihaloethane toxicity.

Small but significant differences in the extent of

alkylation of glycyltryptophan, lysyltyrosine, histidyltyrosine and 2'-deoxyguanosine were observed. The extent of alkylation by S-(2-chloroethyl)-L-cysteine was greater than that for S-(2-chloroethyl)glutathione for the dipeptides just mentioned. The extent of 2'-deoxyguanosine alkylation by S-(2-chloroethyl)glutathione was significantly greater than that of S-(2-chloroethyl)-L-cysteine. These differences in the extent of alkylation were also apparent in the rates of reaction. Histidyltyrosine alkylation by S-(2-chloroethyl)glutathione was 62% of that observed for S-(2-chloroethyl)-L-cysteine whereas glutathione and 2'-deoxyguanosine alkylation by S-(2-chloroethyl)-L-cysteine was 64% and 76% of that for S-(2-chloroethyl)glutathione, respectively. The reason for these differences is not known.

The alkylation of 2'-deoxyguanosine by S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine was reported earlier (Foureman and Reed, 1987). It was observed that S-(2-chloroethyl)-L-cysteine gave rise to detectable amounts of adduct at lower concentrations than S-(2-chloroethyl)glutathione suggesting that it is a better alkylating agent. These data are contradictory to my observations of 2'-deoxyguanosine alkylation. It is not clear what is responsible for the different observations. However, the two experiments did differ in the choice of incubation buffer and the temperature of incubation.

In addition, the interaction of S-(2-chloroethyl)-glutathione with supercoiled plasmid DNA was drastically different than that for the cysteinyl analog (Vadi et al., 1985). The authors had demonstrated that S-(2-chloroethyl)-L-cysteine induces strand relaxation indicative of single strand breaks whereas S-(2-chloroethyl)glutathione did not. The presence of a free alpha-amino group seemed to be

required for plasmid relaxation. However, the mechanism responsible for the observed differences is unknown.

The identification of S-(2-(N⁷-guanylyl)ethyl)-glutathione as the major DNA adduct within the liver and kidneys of 1,2-dibromoethane exposed rats has lead to the suggestion that glutathione sulfur half-mustards play an important role in the carcinogenic potential of the 1,2-dihaloethanes. However, little is known about the relationship between the formation of glutathione sulfur half-mustards *in vivo*, the extent to which these contribute to the total covalent binding to protein and the toxicity that occurs within the liver and kidneys of exposed animals. It was because of this lack of information that this investigation was initiated. The emphasis was to provide a foundation for future investigations to better define the role of glutathione sulfur half-mustards in the above relationships. In this regard, I have demonstrated that cysteinyl thiol groups are particularly good targets for S-(2-chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine alkylation. That other amino acid functional groups are also good targets and that those nucleosides common to DNA do not possess an inherent reactivity which would promote their alkylation over amino acid residues. This characterization of S-(2-chloroethyl)glutathione and it's cysteinyl analog as alkylating agents should benefit future investigations of 1,2-dihaloethane bioactivation and aid in understanding its relationship to toxicity.

1,2-DIHALOETHANE METABOLISM AND GLUTATHIONE DEPLETION IN FRESHLY ISOLATED HEPATOCYTES.

Introduction

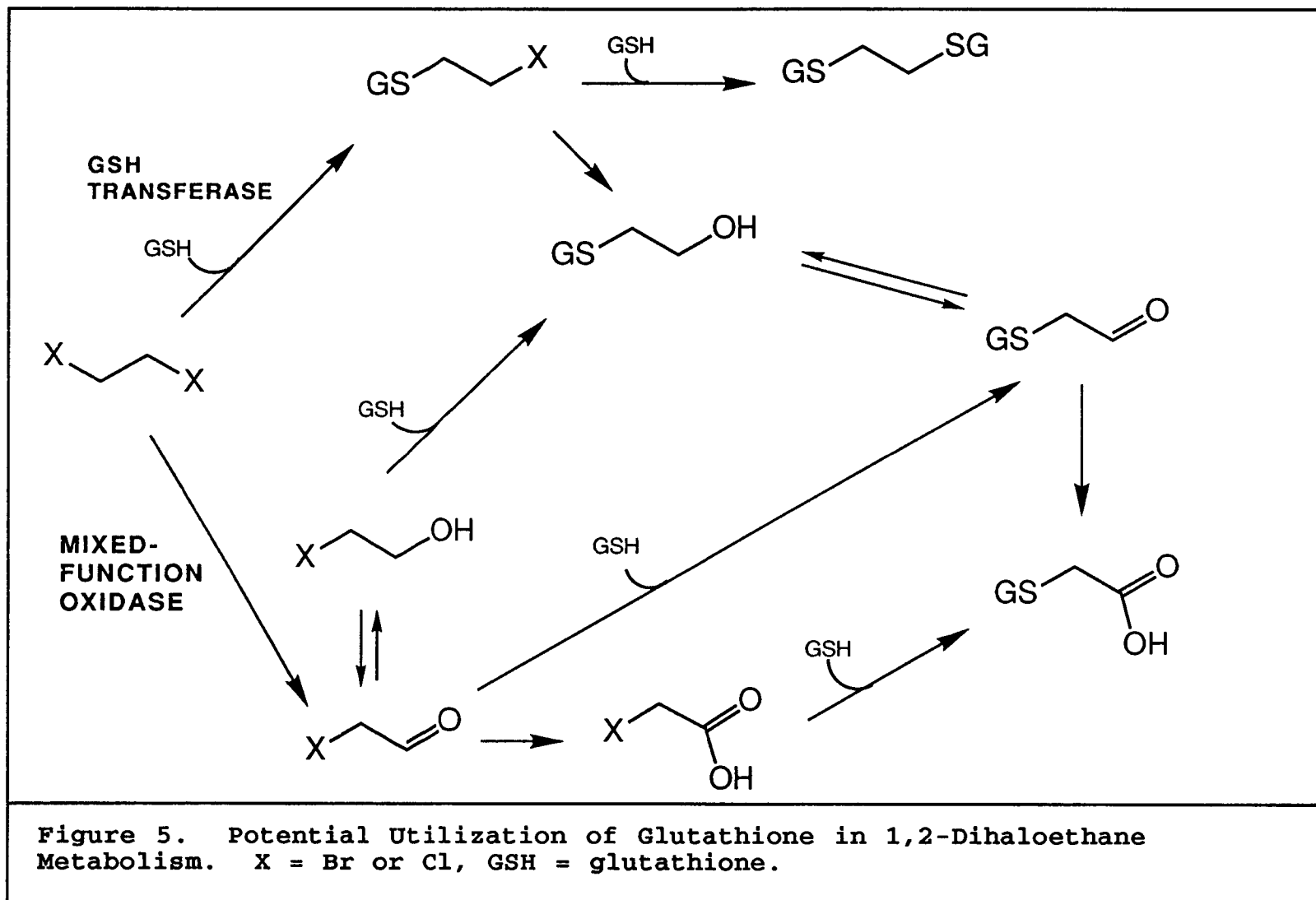
Two distinct metabolic pathways have been proposed as the major routes for 1,2-dihaloethane metabolism in animals (van Bladeren *et al.*, 1981, Guengerich *et al.*, 1980). Each of these pathways can give rise to chemically reactive metabolites and their characterization as alkylating agents has been discussed in the previous chapters. Tissues, such as the liver and kidney, contain substantial amounts of the enzymes responsible for each of the two metabolic pathways. In this regard, they represent major sites for 1,2-dihaloethane metabolism. In addition, the liver and kidneys have been identified as the major target organs for 1,2-dihaloethane toxicity. The covalent binding of 1,2-dihaloethane in these tissues has been shown to be greater than that for other non-target tissues (Arfellini *et al.*, 1984, Hill *et al.*, 1978, Igwe *et al.*, 1986, Reitz *et al.*, 1982). All together these observations suggest a link between metabolism and toxicity.

Glutathione depletion in these tissues is a common characteristic of 1,2-dihaloethane exposure (D'Souza *et al.*, 1988, Mann and Darby, 1985). The depletion of glutathione would seem to complement the finding of large quantities of 1,2-dihaloethane-derived mercapturic acids and other sulfur containing metabolites in the urine of 1,2-dihaloethane-treated animals (Reitz *et al.*, 1982, Yllner, 1971, van Bladeren *et al.*, 1981, Goyal *et al.*, 1989). Presumably each of the urinary products represents the interaction of 1,2-

dihaloethane or a reactive metabolite of 1,2-dihaloethane with glutathione.

The depletion of glutathione from these tissues may be the result of both mixed-function oxidation and glutathione transferase conjugation pathways. Mixed-function oxidation of the 1,2-dihaloethanes produces 2-haloacetaldehydes which may undergo further metabolism to 2-haloethanol and 2-haloacetic acid. The 2-haloacetaldehydes are known to be active alkylating agents toward protein and DNA and may also contribute to glutathione depletion. In addition, direct conjugation of 1,2-dihaloethanes to glutathione, as catalyzed by the glutathione transferases, may contribute both directly and indirectly to glutathione depletion. The S-(2-haloethyl)glutathione produced may react with an additional molecule of glutathione. Figure 5 represents potential sites for the consumption of cellular glutathione in 1,2-dihaloethane metabolism. However, the contributions of either metabolic pathway to the depletion of glutathione and the effect of this on toxicity is yet undefined.

In an effort to further our understanding of the role of glutathione in 1,2-dihaloethane metabolism we have investigated the metabolism of three 1,2-dihaloethanes in freshly isolated hepatocytes. Our particular aim was to characterize changes in intracellular glutathione and to relate these changes to specific routes of metabolism.



Materials and Methods

Chemicals:

Glutathione, gamma-glutamylglutamate, glutathione disulfide, and 1,2-dibromoethane-1,2-¹⁴C (13.3 mCi/mmol) were purchased from Sigma Chemical Company (St. Louis, MO). 1,2-Dichloroethane, 1-Bromo-2-chloroethane, iodoacetamide, 2-chloroethanol, 2-chloroacetic acid and 2-chloroacetaldehyde were purchased from Aldrich Chemical Company (Milwaukee, WI). 1,2-Dibromoethane was purchased from Eastman Kodak Co. (Rochester, NY).

Isolation of Hepatocytes:

Hepatocytes were isolated from male Sprague-Dawley rats (180-210 gm) as described by Fariss et al. (1985). Hepatocyte viability was greater than 90% as assessed by trypan blue exclusion directly after isolation.

Incubation of Hepatocytes with 1,2-Dihaloethanes and Chlorinated Derivatives:

Freshly isolated hepatocytes were resuspended to a concentration of 2.0×10^6 cells per ml in a modified Fischer's medium which lacked all sulfur amino acids except for methionine (0.67 mM) and contained 3.5 mM calcium, 10 mM 4-((2-hydroxyethyl)-1-piperazine)ethanesulfonic acid, and 20-60 mM gamma-glutamylglutamate as an internal standard for HPLC analysis. Ten milliliters of cell suspension was placed into 25 ml Erlenmeyer flasks and an aliquot of agent added. The 1,2-dihaloethanes were added directly to the flasks whereas the chlorinated derivatives were prepared in water just prior to addition. The flasks were sealed with rubber septums and incubated at 37°C for one hour in an

orbital shaker. After incubation, aliquots of the cell suspension were taken and prepared for high performance liquid chromatography (HPLC) analysis.

Studies with the radiolabeled 1,2-dibromoethane were conducted as described above for the nonradiolabeled 1,2-dihaloethanes. However, the eluent from the HPLC ultraviolet detector was collected in one minute fractions and mixed with Formula 969 scintillation cocktail (NEN Research Products, Boston, Mass.). The radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer. The quenching of samples was considerable and required that each vial be counted twice, before and after spiking with ^{14}C -toluene.

Lactate dehydrogenase leakage was determined as described by Fariss et al. (1985) except that a Kontron spectrophotometer (UVikon 810) was used in place of the Beckman Enzyme Activity Analyzer.

HPLC Analysis:

To quantitate the formation of glutathione-containing metabolites, 0.6 ml of cell suspension was added to 0.1 ml 70% perchloric acid (PCA) in 1.5 ml microfuge tubes. After vortexing and centrifugation (13000 x g for one minute) an aliquot of the supernatant was derivatized with Sanger's reagent as described (Fariss and Reed, 1987) except that iodoacetamide was substituted for iodoacetic acid.

Determination of intracellular glutathione was performed as described by Fariss and Reed (1987) with the noted exception. Briefly, 0.6 ml of cell suspension was placed over 0.4 ml of dibutylphthalate that was layered over 0.5 ml 10% PCA containing 20 nmoles gamma-glutamylglutamate. Centrifugation for one minute at 13000 x g was used to pellet "viable" cells through the oil layer and into the

PCA. Aliquots of the PCA supernatant were assayed as above.

HPLC employed anion exchange columns prepared from 3-aminopropylsilane-derivatized Spherisorb, a binary mobile phase system consisting of 80% methanol and 0.8 M acetate in 80% methanol as described (Jean and Reed, 1989). Note that longer columns (4.6 mm x 300 mm) and a variety of gradient programs were used to aid in the resolution of components. Standards of the compounds of interest were derivatized as above for the cell samples and their retention times characterized with each column/gradient system just prior to analysis of cell samples. A SpectraPhysics HPLC pump and SP8700XR pump controller, Micrometrics autosampler model #728, Spectroflow 757 UV detector and Hewlett Packard integrator model 3390A were used.

Chemical Synthesis:

The synthesis of S-(2-hydroxyethyl)glutathione was carried out by adding excess 1-bromoethanol to glutathione under alkaline conditions. Isolation of S-(2-hydroxyethyl)-glutathione from the reaction mixture was performed by preparative HPLC using a reverse phase C18 column (Whatman ODSII, 20mm x 500mm) and 0.1% acetic acid as the mobile phase. S-(2-Hydroxyethyl)glutathione was collected upon elution from the column, frozen, lyophilized and characterized by Fast Atom Bombardment mass spectrometry. Purity was judged to be better than 97% by HPLC with the major contaminate being glutathione.

S,S'-(1,2-Ethanediy1)-bis[glutathione] was formed by mixing two equivalences of glutathione with one equivalent of 1,2-dibromoethane in 30% ethanol in the presence of triethanolamine, pH = 9.0 at room temperature for 24 hours (Nachtomi, 1970). Isolation and characterization of S,S'-(1,2-ethanediy1)bis[glutathione] from the reaction mixtures

was performed as above with the exception of using 10% methanol, 0.1% acetic acid as the mobile phase.

S-Carboxymethyl-glutathione was isolated from the reaction mixture in which 100 mg of glutathione was added to 120 mg iodoacetic acid in 5.0 ml 0.5 M Tris-base at pH = 9.1. Preparative HPLC was used to purify the S-carboxymethyl-glutathione as was done for S-(2-hydroxyethyl)-glutathione.

Glutathione reacts readily with 2-chloroacetaldehyde to form S-formylmethyl-glutathione (Johnson, 1967). However, the isolation of S-formylmethyl-glutathione from its reaction mixture was unsuccessful. Our attempts to isolate S-formylmethyl-glutathione from reactions mixtures were also unsuccessful.

Results

Exposure of freshly isolated hepatocytes to 1.2 mM 1,2-dichloroethane, 1-bromo-2-chloroethane, or 1,2-dibromoethane gave rise to various levels of glutathione depletion by one hour (Figure 6). The 1,2-dihaloethane-induced loss of intracellular glutathione was greatest for 1,2-dibromoethane (83% depletion) and least for 1,2-dichloroethane (19% depletion). The loss of glutathione from these cells was not due to cell lysis as suggested by the lack of lactate dehydrogenase leakage from treated cells (data not shown).

Exposure of freshly isolated hepatocytes to radio-labeled 1,2-dibromoethane gave rise to the formation of at least three metabolites with retention times characteristic of S-(2-hydroxyethyl)glutathione, S-carboxymethyl-glutathione and S,S'-(1,2-ethanediy)bis[glutathione] (Figure 7).

The amount of each metabolite formed for the different 1,2-dihaloethanes varied as shown in figure 8. 1,2-Dibromoethane and 1-bromo-2-chloroethane gave rise to large amounts of S-(2-hydroxyethyl)-glutathione. The amount of S-carboxymethyl-glutathione was very low for all three of the 1,2-dihaloethanes whereas the amount of S,S'-(1,2-ethanediy)bis[glutathione] varied. As with the depletion of glutathione, the level of S,S'-(1,2-ethanediy)bis[glutathione] formed was directly proportional to the bromine content, ie. the greater the bromine content the greater the amount of S,S'-(1,2-ethanediy)bis-[glutathione] formed.

There were a total of five different forms of glutathione measured in this study, glutathione, glutathione disulfide, S-(2-hydroxyethyl)glutathione, S-carboxymethyl-glutathione and S,S'-(1,2-ethanediy)bis[glutathione]. The

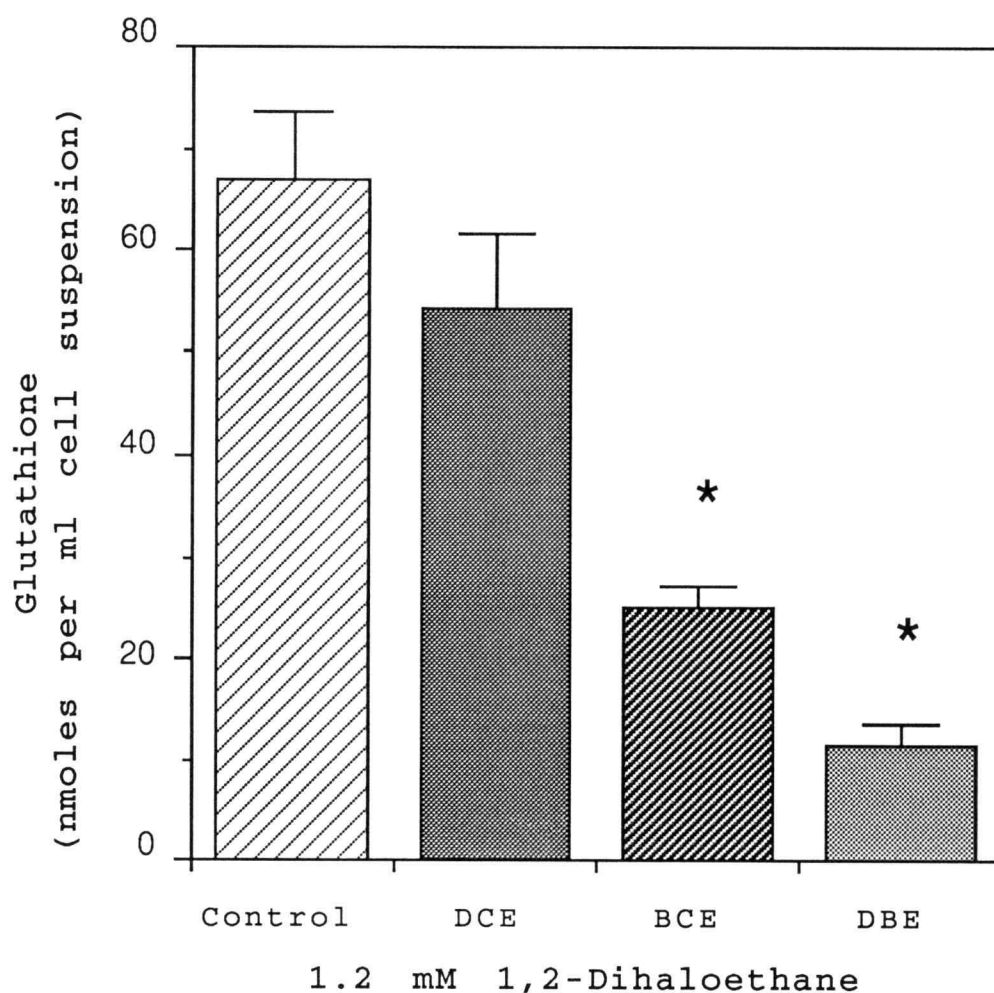


Figure 6. 1,2-Dihaloethane-induced Depletion of Glutathione in Isolated Rat Hepatocytes. Hepatocytes (2.0×10^6 cells per ml) were treated with 1,2-dihaloethane for one hour and the amount of glutathione remaining was determined as described in the "Materials and Methods" section. Values represent the mean of at least three to five experiments \pm SEM. (*) denotes significantly different from control ($p < 0.01$). DCE = 1,2-dichloroethane, BCE = 1-bromo-2-chloroethane and DBE = 1,2-dibromoethane.

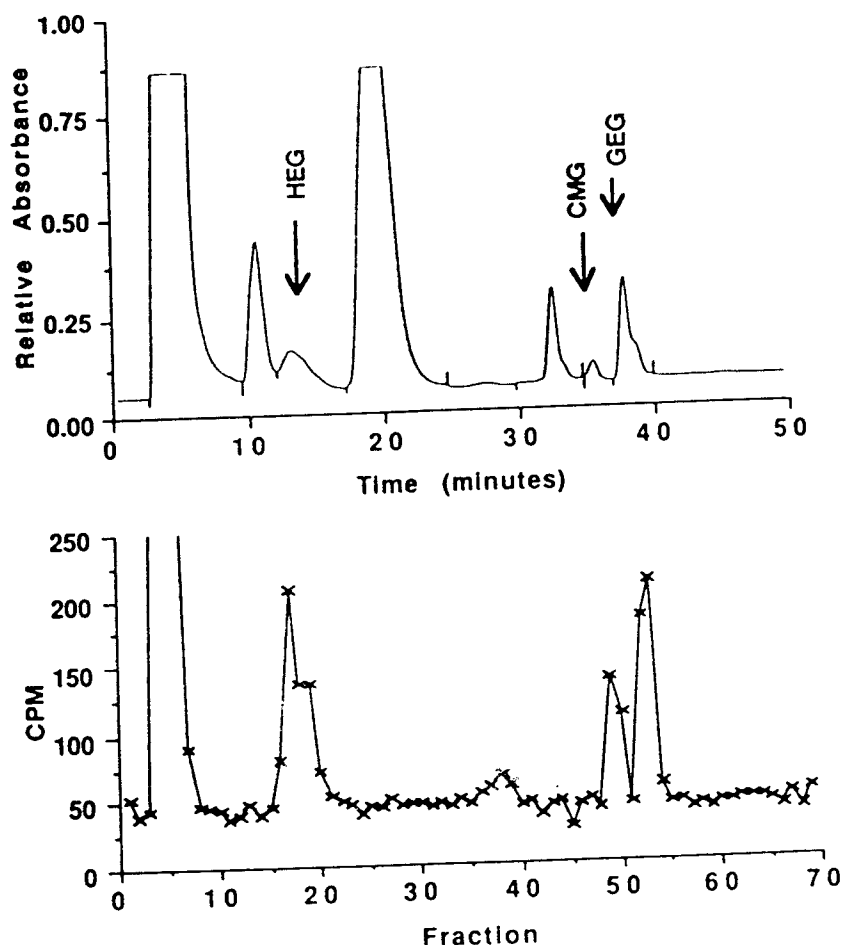


Figure 7. HPLC Analysis of Glutathione-containing Metabolites from 1,2-Dibromo-[1,2- ^{14}C]-ethane Treated Rat Hepatocytes. The upper chromatogram represents the elution of 365 nm absorbing material and the lower chromatogram radioactivity. Freshly isolated hepatocytes were treated and assayed as described in the "Materials and Methods" section. Peak assignments are based upon the elution times of standards. HEG = S-(2-hydroxy-ethyl)glutathione, CMG = S-2-carboxymethyl-glutathione, and GEG = S,S'-(ethanediy)bis-[glutathione].

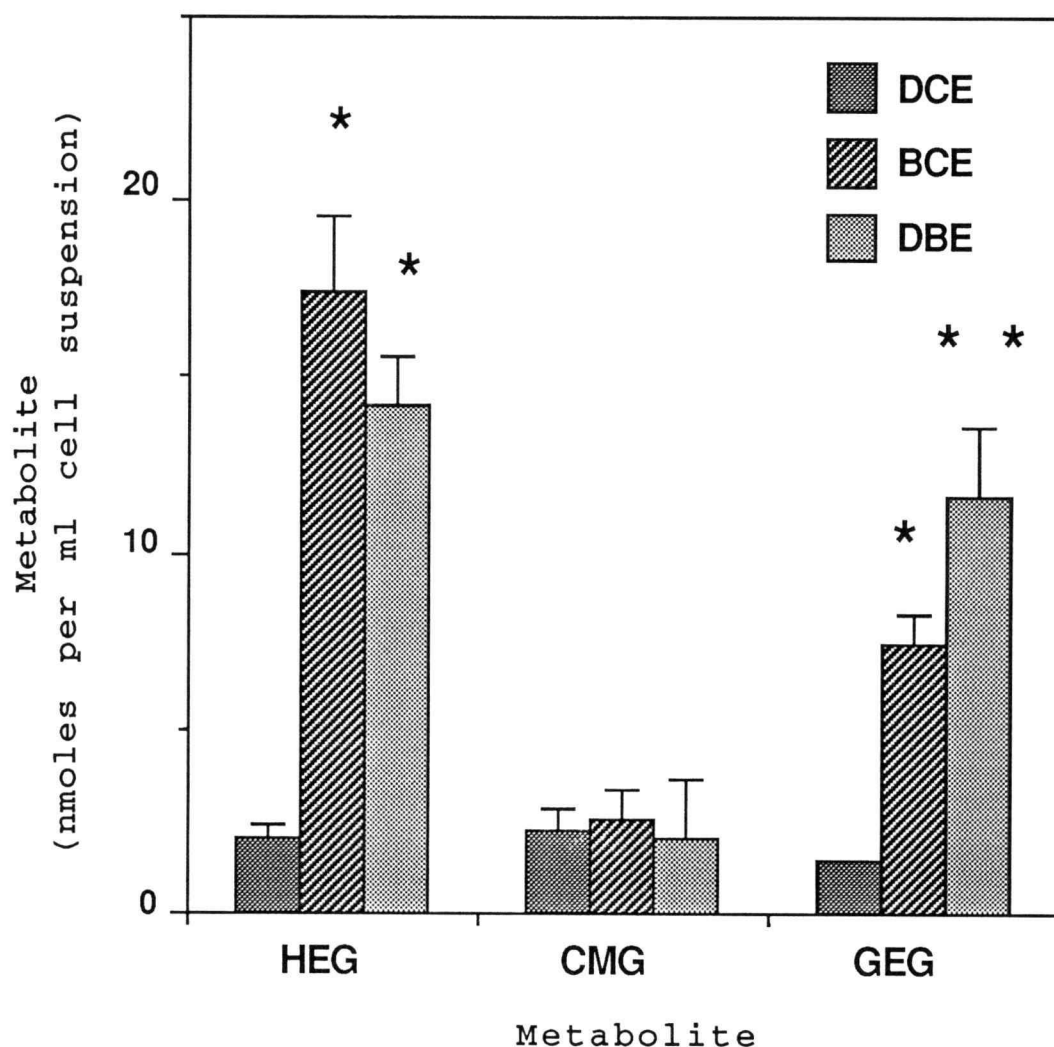


Figure 8. Quantitation of Glutathione-containing Metabolites from 1,2-Dihaloethane Treated Rat Hepatocytes. Freshly isolated hepatocytes were treated with 1.2 mM 1,2-dihaloethane for one hour as described in the "Materials and Methods" section. Values represent the mean of at least five separate experiments \pm SE. (*) Significantly different from DCE ($p < 0.01$) and (**) significantly different than DCE and BCE ($p < 0.05$). DCE = 1,2-dichloroethane, BCE = 1-bromo-2-chloroethane, DBE = 1,2-dibromoethane, HEG = S-(2-hydroxyethyl)-glutathione, CMG = S-carboxymethyl-glutathione, GEG = S,S'-(1,2-ethanediy1)bis[glutathione].

levels of each are depicted in figure 9 as the equivalents of glutathione found in each. These data demonstrate that 55% or more of the depleted glutathione could be accounted for in the formation of the three glutathione-containing metabolites. The only significant loss of glutathione that could not be accounted for occurred with 1,2-dibromoethane. Approximately 20% of the glutathione loss could not be accounted for, an observation that led to the examination of 1,2-dibromoethane covalent binding.

In addition to glutathione depletion, 1,2-dibromoethane became covalently bound to hepatocellular protein (Table 10). The rate of 1,2-dibromo-[1,2- ^{14}C]-ethane covalent binding decreased with the time of incubation and correlated with glutathione depletion. After 2 hours of incubation, a total of 18.7 nmoles of 1,2-dibromo-[12- ^{14}C]-ethane was covalently bound per ml cell suspension. Half of this total occurred within the first 30 minutes of incubation (9.6 nmoles per ml cell suspension). Intracellular glutathione remained relatively high during this same period remaining above 30% of initial levels. By 60 minutes of incubation, 77% of the total covalent binding occurred under conditions of glutathione depletion to only 17% of initial levels. The second hour of incubation gave rise to almost complete depletion of intracellular glutathione accompanied by an additional amount of covalent binding of only 4.3 nmoles per ml cell suspension.

In an effort to understand how the mixed-function oxidase metabolites may contribute to the loss of glutathione and the formation of these glutathione-containing metabolites, freshly isolated hepatocytes were treated with 2-chloroacetaldehyde, 2-chloroethanol and 2-chloroacetic acid. The amount of S-(2-hydroxyethyl)-glutathione, S-carboxymethyl-glutathione and S,S'-(1,2-

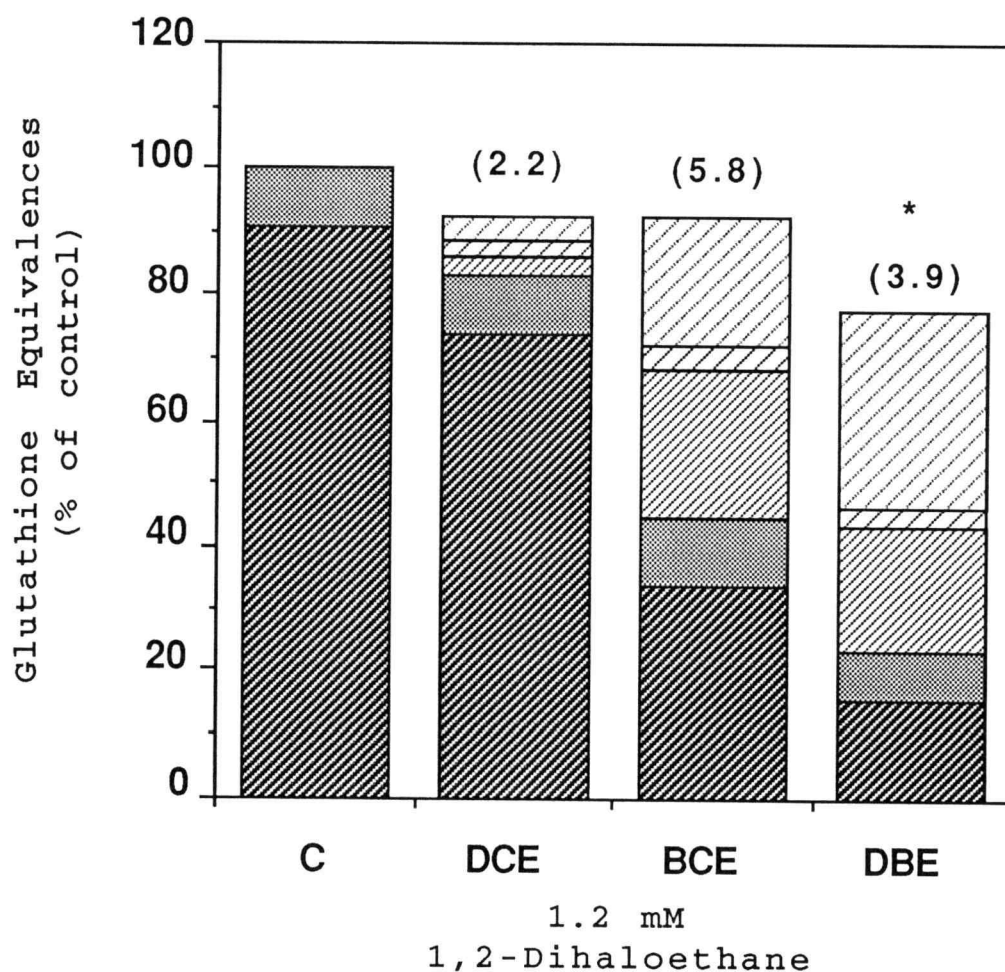


Figure 9. Accountability of 1,2-Dihaloethane-induced Depletion of Glutathione. The quantity of glutathione recovered as glutathione (GSH), glutathione disulfide (GSSG), S-(2-hydroxyethyl)glutathione (HEG), S-carboxymethyl-glutathione (CMG), and S,S''-(ethandiyl)bis[glutathione] (GEG) following 1 hour of incubation with 1.2 mM 1,2-dihaloethane. Data are expressed as the percent of glutathione equivalences for control. Values represent the mean (\pm SEM) of at least three separate experiments. "*" denotes "significantly different than control" ($p < 0.05$). C = control, DCE = 1,2-dichloroethane, BCE = 1-bromo-2-chloroethane, DBE = 1,2-dibromoethane.

GSH (▨), GSSG (▩), HEG (□), CMG (▤), GEG (▥)

Table 10. Time-dependent Accumulation of Glutathione-containing Metabolites and Covalent Binding During 1,2-Dibromo-[1,2-¹⁴C]-ethane Metabolism by Isolated Rat Hepatocytes.

	Control	Treated					Protein Covalent Binding ^a	Total
Time [minutes]	GSH	GSH [nmoles	HEG GSH	CMG equivalence	GEG per ml	cell	suspension(±SE)]	
0	45.2 (8.1)	45.2 (8.1)	0.0	0.0	0.0	0.0	0.0	45.2
15	47.3 (7.6)	30.6 (3.2)	4.9 (0.7)	3.5 (3.5)	10.6 (3.9)	5.3 (0.6)	5.3	54.9
30	52.8 (8.8)	15.2 (3.5)	9.2 (1.4)	6.2 (0.9)	14.6 (3.7)	9.6 (0.7)	9.6	54.8
60	65.5 (9.8)	8.1 (1.2)	14.1 (2.2)	8.3 (1.6)	16.4 (5.9)	14.4 (0.8)	14.4	61.3
120	104.1 (20.2)	3.2 (3.9)	8.4 (2.9)	11.1 (7.4)	21.2 (7.0)	18.7 (1.8)	18.7	83.3

These data represent at least three separate experiments. ^anmoles of covalently bound 1,2-dibromoethane per ml cell suspension based upon an estimated 2.4 mg protein per ml cell suspension (2.0 x 10⁶ cells per ml). GSH = glutathione, HEG S-(2-hydroxyethyl)glutathione, CMG = S-2-carboxymethyl-glutathione, GEG = S,S'-(1,2-ethanediy)bis-[glutathione].

ethanediyl)bis[glutathione] were determined (Table 11). None of these compounds gave rise to the formation of S,S'-ethylene-bis-glutathione. 2-Chloroacetic acid gave rise solely to S-carboxymethyl-glutathione where as 2-chloroethanol produced principally S-carboxymethyl-glutathione (93%) and only a small amount of S-(2-hydroxyethyl)glutathione (7%). 2-Chloroacetaldehyde produced both S-(2-hydroxyethyl)glutathione (44%) and S-carboxymethyl-glutathione (56%).

1,2-Dihaloethane incubations were also performed under conditions of high extracellular glutathione (1.0 mM) to determine the extent to which glutathione sulfur half-mustards exit the cells. The presence of extracellular glutathione was found to increase the amount of S,S'-(1,2-ethanediyl)bis[glutathione] by 120, 179 and 161% following incubation with 1,2-dichloroethane, 1-bromo-2-chloroethane and 1,2-dibromoethane, respectively (Table 12). The effect of extracellular glutathione on S-(2-hydroxyethyl)glutathione was less dramatic and varied with the 1,2-dihaloethane.

S-Carboxymethyl-glutathione formation was not altered by the presence of extracellular glutathione.

Table 11. Metabolism of 1,2-Dichloroethane Derivatives to Glutathione-containing Metabolites.

Agent	HEG (nmoles per ml cell suspension \pm SE)	CMG	GEG
2-chloroacetic acid	ND	49.0 \pm 6.9	ND
2-chloroethanol	3.1 \pm 1.0	35.8 \pm 3.0	ND
2-chloroacetaldehyde	23.0 \pm 2.6	29.2 \pm 2.8	ND

Freshly isolated hepatocytes were treated with 2-chloroethanol (0.5 mM), 2-chloroacetic acid (0.5 mM), and 2-chloroacetaldehyde (0.25 mM) for 1 hour, and the amount of glutathione-containing metabolites present determined. Values represent the mean \pm SE for at least three separate experiments. "ND" = formation of metabolite was not detected.

HEG = S-(2-hydroxyethyl)glutathione

CMG = S-carboxymethyl-glutathione

GEG = S,S'-(1,2-ethandiyl)bis(glutathione)

Table 12. Effect of Extracellular Glutathione (1.0 mM) on the Formation of S,S'-(1,2-Ethanediy1)bis[glutathione].

Agent	GEG (% of Control \pm SE)
1,2-dichloroethane	120.5 \pm 22.4
1-bromo-2-chloroethane	179.3* \pm 14.1
1,2-dibromoethane	161.4* \pm 12.3

Freshly isolated hepatocytes were treated with 1.2 mM 1,2-dihaloethane in the presence or absence (control) of 1.0 mM glutathione and the quantity of S,S'-(1,2-ethanediy1)-bis[glutathione] (GEG) determined. The values represent the effective change in metabolite formation by the inclusion of extracellular glutathione. The values represent the mean \pm SE for at least three separate experiments. (*) The amount of metabolite formed with the addition of extracellular glutathione was significantly different ($p < 0.05$) than control.

Discussion

This investigation has demonstrated that exposure of freshly isolated hepatocytes to any one of three 1,2-dihaloethanes results in the loss of intracellular glutathione with the concomitant production of at least three glutathione-containing metabolites. The ability of these 1,2-dihaloethanes to reduce intracellular glutathione in this model system is characteristic of these same agents *in vivo* (D'Souza *et al.*, 1988, Mann and Darby, 1985).

As depicted in figure 2, the metabolism of 1,2-dihaloethanes to reactive species could occur through two different pathways. Glutathione, as the major nonprotein thiol in these cells, could potentially participate in 1,2-dihaloethane metabolism in a variety of ways as outlined in figure 5. Our investigation has shown that S-(2-hydroxyethyl)glutathione, S-carboxymethyl-glutathione and S,S'-(1,2-ethanediyl)bis[glutathione] are produced following exposure of freshly isolated hepatocytes to 1,2-dichloroethane, 1-bromo-2-chloroethane and 1,2-dibromoethane. Presently there remains considerable uncertainty as to the contributions of either pathway in the production of S-(2-hydroxyethyl)-glutathione and S-carboxymethyl-glutathione. Both could be produced from either the mixed-function oxidase or the glutathione transferase pathway. However, S,S'-(1,2-ethanediyl)bis[glutathione] formation is thought to originate solely from the glutathione S-transferase pathway.

The metabolism of various putative mixed-function oxidase metabolites of 1,2-dichloroethane was studied in an effort to determine their ability to produce the same glutathione-containing metabolites as was formed with the

1,2-dihaloethanes. 2-Chloroacetaldehyde gave rise to almost equal amounts of S-(2-hydroxyethyl)glutathione and S-carboxymethyl-glutathione. Given that 2-chloroacetaldehyde reacts rapidly with glutathione *in vitro* (Johnson, 1965) it is assumed that upon entering the hepatocytes it would quickly react with intracellular glutathione to form S-formylmethyl-glutathione. Attempts to purify and characterize S-formylmethyl-glutathione were unsuccessful as it appears that this compound is not stable under the conditions used for HPLC derivatization and analysis. Nonetheless, the possibility remains that the intracellular formation of S-formylmethyl-glutathione may result in the formation of S-carboxymethyl-glutathione or S-(2-hydroxyethyl)glutathione via further oxidation or reduction, respectively.

The data obtained suggest that both conversions are possible and that neither is favored over the other resulting in almost equal amounts of S-(2-hydroxyethyl)-glutathione and S-carboxymethyl-glutathione formation. These data suggest that 2-chloroethanol, which gave rise primarily to S-carboxymethyl-glutathione, was oxidized to the carboxylic acid prior to its conjugation with glutathione. The amount of S,S'-(1,2-ethanediyl)bis-[glutathione] formed from the 1,2-dihaloethanes may be taken as an indirect measure of sulfur half-mustard formation within the hepatocytes. This would be an estimate of the lowest level of mustard formation because alkylation of glutathione is but one of the various reactions that the sulfur half-mustards may undergo. The sulfur half-mustards are known to be unstable, giving rise to S-(2-hydroxyethyl)-glutathione as well as to react with protein and nucleic acids. Thus, it is possible that hydrolysis of the sulfur half-mustards could be responsible for a large proportion of

the total S-(2-hydroxyethyl)glutathione formed in these experiments. This would seem to be the true based upon the observations related to the metabolism of 2-chloroacetaldehyde. S-(2-hydroxyethyl)glutathione formation from 1,2-dihaloethane mixed-function oxidation should be no greater than the amount of S-carboxymethyl-glutathione formation. The amount of S-carboxymethyl-glutathione formation was very low for all of the 1,2-dihaloethane exposures. This would suggest that the majority of the S-(2-hydroxyethyl)glutathione produced be due to the hydrolysis of the respective S-(2-haloethyl)glutathione. Therefore, hepatocytes may be exposed to a substantial amount of sulfur half-mustards, particularly in the case of 1,2-dibromoethane and 1-bromo-2-chloroethane. Apparently the glutathione transferase pathway is responsible for the majority of the 1,2-dihaloethane-induced glutathione depletion.

The ability of hepatocytes to export of glutathione and various glutathione S-conjugates is well known (Sies, 1983, Ormstad and Orrenius, 1983). The mutagenicity of bile from 1,2-dichloroethane-treated rats has been attributed to the hepatocellular excretion of S-(2-chloroethyl)glutathione into the bile (Rannug and Beije, 1979). More recently, S-(2-chloroethyl)glutathione has been identified in the bile of 1-bromo-2-chloroethane (Marchand and Reed, 1989). Altogether, these data indicate that 1,2-dihaloethane-treated hepatocytes may potentially excrete S-(2-haloethyl)-glutathione during 1,2-dihaloethane metabolism. To assess this possibility, the effect of extracellular glutathione on the formation of S,S'-(1,2-ethanediyl)bis[glutathione] following 1,2-dihaloethane exposure was examined. Extracellular glutathione was found to significantly increase the formation of S,S'-(1,2-ethanediyl)bis-

[glutathione] from 1-bromo-2-chloroethane and 1,2-dibromoethane. These findings are in agreement with the proposed ability of hepatocytes to excrete the glutathione sulfur half-mustards.

It is apparent from the metabolism studies that not all of the glutathione depletion could be accounted for with the formation of the three glutathione-containing metabolites. This was especially true for 1,2-dibromoethane in which approximately 21.4 nmoles of glutathione per ml cell suspension could not be accounted for following 1 hour of incubation. The relative amounts of glutathione-containing metabolites formed and the investigations with 2-chloroacetaldehyde strongly suggest that the glutathione transferase catalyzed conjugation of 1,2-dibromoethane with glutathione, to form S-(2-bromoethyl)glutathione, was responsible for the majority of the glutathione depletion and metabolite formation. Therefore, it is possible that the loss of glutathione may be due to the alkylation of cellular macromolecules by S-(2-bromoethyl)glutathione. To investigate this further, hepatocytes were treated with 1,2-dibromo-[1,2-¹⁴C]-ethane and the amount of 1,2-dibromo-[1,2-¹⁴C]-ethane covalently bound to hepatocyte protein determined. The greatest extent of covalent binding occurred under conditions of high intracellular glutathione. In terms of accountability, the total amount of glutathione present as glutathione and glutathione-containing metabolites when added to the amount of covalent binding, agrees well with the total amount of glutathione in controls. These data suggest an interesting relationship between the formation of glutathione sulfur half-mustards and covalent binding that may be important to 1,2-dihaloethane toxicity.

Overall this investigation has shown that freshly

isolated hepatocytes are susceptible to 1,2-dihaloethane-induced glutathione depletion as is characteristic of the liver in 1,2-dihaloethane-treated animals. The majority of the glutathione depletion can be accounted for in the formation of three glutathione-containing metabolites. In addition, these data suggest that the glutathione transferase catalyzed conjugation of 1,2-dihaloethanes to glutathione is responsible for the majority of the glutathione depletion. This would represent the formation of substantial amounts of glutathione sulfur half-mustards. However, the importance of the glutathione sulfur half-mustard formation in 1,2-dihaloethane-induced toxicity has yet to be clearly defined.

1,2-DIBROMOETHANE-INDUCED TOXICITY IN FRESHLY ISOLATED RAT HEPATOCYTES

Introduction

1,2-Dihaloethanes are widely used in the world today and have become environmental contaminants of some concern. They are mutagenic (Fabricant and Chalmers, 1980, Hooper *et al.*, 1980), carcinogenic in laboratory animals (NCI, 1978, Weisburger, 1977) and toxic to humans and laboratory animals (WHO, 1987, van Bladeren *et al.*, 1981, Guengerich *et al.*, 1980). The available data strongly implicate metabolism in the mutagenicity and carcinogenicity of these compounds (Rannug *et al.*, 1978, van Bladeren *et al.*, 1980, Rannug, 1980). Specifically, metabolism involving glutathione conjugation and the formation of reactive sulfur half-mustards.

Glutathione sulfur half-mustards are unique in that they denote the unusual participation of glutathione in xenobiotic bioactivation. The glutathione sulfur half-mustard, S-(2-chloroethyl)glutathione, has been shown to be a direct-acting alkylating agent (Jean and Reed, 1989) and recent studies have demonstrated its formation *in vivo* (Marchand and Reed, 1989). S-(2-Chloroethyl)glutathione and its derivatives have been shown to be mutagenic (Humphreys *et al.*, 1990). In addition, S-(2-(N⁷-guanylyl)ethyl)-glutathione was identified as the major DNA adduct in rat liver and kidney following 1,2-dibromoethane exposure (Koga *et al.*, 1986, Inskeep *et al.*, 1986).

1,2-Dihaloethane exposures of laboratory animals and humans have demonstrated a variety of effects concerning

central nervous system depression and injury to the exposed tissues and internal organs (WHO, 1987, Alexeeff *et al.*, 1990). The major target organs of 1,2-dihaloethane toxicity have been identified as the liver and kidney. The mechanism(s) responsible for the target organ toxicities have not been defined. Difficulties with assessing toxicity in *in vivo* systems coupled with the presence of two distinct metabolic pathways for 1,2-dihaloethane metabolism, both of which produce reactive metabolites, have greatly limited progress towards elucidation of the mechanisms involved.

Investigations *in vivo* have demonstrated that the liver of 1,2-dihaloethane exposed animals undergoes a dramatic loss of glutathione shortly after exposure (Botti *et al.*, 1982, Johnson, 1965). That covalent binding of 1,2-dihaloethane to protein, RNA and DNA in the liver and kidney is greater than that for any of the other tissues studied (Arfellini *et al.*, 1984, Reitz *et al.*, 1982, Hill *et al.*, 1978). In addition, a variety of urinary metabolites have been identified (WHO, 1987, van Bladeren *et al.*, 1981), most containing sulfur thought to be derived from glutathione.

Prior studies from our laboratory have demonstrated that isolated hepatocytes metabolize 1,2-dichloroethane, 1-bromo-2-chloroethane and 1,2-dibromoethane to at least three glutathione-containing metabolites (Jean and Reed, 199X). These metabolites could not account for the whole of glutathione depletion with the greatest deficit occurring for 1,2-dibromoethane. The amount of covalent binding of 1,2-dibromoethane to cellular protein was also studied and found to be in close agreement with that amount of glutathione unaccounted for. These data, together with what is known of the reactivity of glutathione sulfur half-mustards and of their potential for formation, strongly suggest that the covalent binding of 1,2-dibromoethane to

hepatocellular protein was the result of S-(2-bromoethyl)-glutathione formation.

However, the importance of glutathione sulfur half-mustard formation in 1,2-dihaloethane-induced hepatotoxicity is yet uncertain. Conjugation of glutathione with 1,2-dihaloethanes would seem to promote a hazardous situation for cells by producing reactive alkylating agents concomitant with depletion of intracellular glutathione. To investigate this relationship further, the effect of modulating intracellular glutathione on the toxicity of 1,2-dibromoethane in freshly isolated hepatocytes was studied. This report details my findings as related to glutathione depletion, lipid peroxidation and 1,2-dibromoethane-induced cell death in freshly isolated rat hepatocytes.

Materials and Methods

Chemicals:

1,2-Dibromoethane, methionine, N-acetyl-cysteine, iodoacetamide, butylated hydroxyanisole, ruthenium red, and William's E medium were purchased from Sigma Chemical Company (St. Louis, Mo.). Desferal mesylate was kindly provided by Ciba-Geigy (Summit, NJ). All other chemicals were reagent grade or better.

Isolation of Hepatocytes and Modulation of Intracellular Glutathione Levels:

Hepatocytes were isolated from male Sprague-Dawley rats (180-200gm) as described (Fariss and Reed, 1987) with slight modification as noted below. Isolation of hepatocytes as described by Fariss and Reed (1987) produced hepatocytes containing approximately 14 nmoles of glutathione per million cells. Viability of hepatocytes directly following isolation was better than 90% as assessed by trypan blue exclusion. The substitution of William's E medium (serum free) for Hank's bicarbonate buffer during collagenase perfusion and suspension of isolated hepatocytes in Fischer's medium containing 0.67 mM methionine gave rise to hepatocytes with elevated levels of glutathione (23 to 45 nmoles per million cells). As above, viability was greater than 90%.

The term "system-1" will denote throughout this report the isolation and incubation of hepatocytes in the absence of sulfur amino acids and "system-2" for the isolation and incubation in the presence of sulfur amino acids.

Incubation of Hepatocytes with 1,2-Dibromoethane:

Hepatocytes obtained following the Hank's/collagenase perfusion were suspended (2.0×10^6 cell per ml) in Fisher's media containing 2.5 mM calcium, 10 mM 4-((2-hydroxyethyl)-1-piperazine)ethanesulfonic acid (HEPES) and lacking all sulfur amino acids (Fariss and Reed, 1987). Twenty milliliters of cell suspension were placed into 50-ml Florence flasks and 1,2-dibromoethane added directly into each flask with dimethylsulfoxide as the vehicle (0.1 ml dimethylsulfoxide per 20 ml of cell suspension). The flasks were sealed with rubber septums immediately after addition. Cells were then incubated for 6 hours at 37°C in an orbital shaker. The cell suspensions were incubated under a constant flow of 95% O₂, 5% CO₂ after the first hour of incubation.

Hepatocytes isolated following William's E medium/collagenase perfusion were treated as above with one exception; methionine (0.67 mM) was included in the Fischer's medium.

Addition of Various Agents:

The following agents (0.1 ml) were added to the appropriate flasks after the first hour of incubation with 1,2-dibromoethane. Desferal mesylate (40 μ moles/ ml 20mM HEPES), ruthenium red (5 μ moles/ ml 20 mM HEPES) or butylated hydroxyanisole (20 μ moles/ml dimethylsulfoxide) were added to flasks containing 20 ml of cell suspension to yield concentrations of 0.2 mM, 25 μ M, and 0.1 mM, respectively. N-Acetyl-cysteine was added as the dry powder to the cell suspension yielding a concentration of 10 mM.

Determination of Intracellular Glutathione and Formation of Thiobarbituric Acid-reactive Species:

The determination of intracellular glutathione was performed as described (Fariss and Reed, 1987). However, iodoacetamide was substituted for iodoacetic acid in the derivatization of glutathione prior to the addition of Sanger's reagent.

The amount of thiobarbituric acid-reactive species present in 0.25 ml cell suspension was determined as described (Stacey and Klaassen, 1981). Aliquots of sample were placed into 96-well plates for colorimetric analysis utilizing a multi-well plate reader (#EL340, Bio-Tek Instruments Inc., Winooski, VT).

Determination of Lactate Dehydrogenase Leakage:

Lactate dehydrogenase activity was assayed spectrophotometrically. An aliquot of medium devoid of cells was diluted with an equal volume of saline. Ten microliters of this diluted media was placed into the wells of a 96-well plate. Two hundred microliters of lactate dehydrogenase reagent (Beckman Liquid-Stat LD-L reagent, Beckman Instruments Inc., Brea, CA) was then added and the increase in absorbance at 340 nm recorded with a multi-well plate reader. The rate of change of absorbance was compared with the total lactate dehydrogenase activity present in the cell suspensions upon initiation of the experiment. Total lactate dehydrogenase activity was determined as above following addition of 150 μ l of cell suspension to 50 μ l of 0.4% Triton X-100, brief sonication (5 seconds) and addition of 0.4 ml of saline.

Results

Intracellular glutathione was found to differ markedly depending upon the isolation procedure and incubation medium (Figure 10). The isolation and incubation of hepatocytes in the presence of media containing sulfur amino acids gave rise to elevated intracellular glutathione at the start of each experiment with continued elevation during the 6 hours of incubation to levels four times greater than isolation and incubation without sulfur amino acids.

The toxicity of 1,2-dibromoethane (100, 200 and 500 μM), as assessed by leakage of lactate dehydrogenase, was markedly different under conditions of elevated versus reduced intracellular glutathione (Figure 11). Within 2 hours of exposure to 200 and 500 μM 1,2-dibromoethane, hepatocytes in system-1 had expressed significant leakage of lactate dehydrogenase. By 6 hours this leakage amounted to greater than 75% of total. 1,2-Dibromoethane (100 μM) exposed cells exhibited significant lactate dehydrogenase leakage only after 6 hours of incubation. In contrast, 100, 200 and 500 μM 1,2-dibromoethane did not induce significant lactate dehydrogenase leakage from hepatocytes in system-2 during the course of incubation.

1,2-Dibromoethane induced a dose- and time-dependent depletion of intracellular glutathione for both system-1 and system-2 hepatocytes (Figure 12). However, the degree of depletion and subsequent repletion were dramatically different between the two systems. Under system-1 conditions, intracellular glutathione was depleted to below 5 nmoles of glutathione per million cells within the first hour of incubation. Within 4 and 6 hours of incubation intracellular glutathione was depleted to below detectable

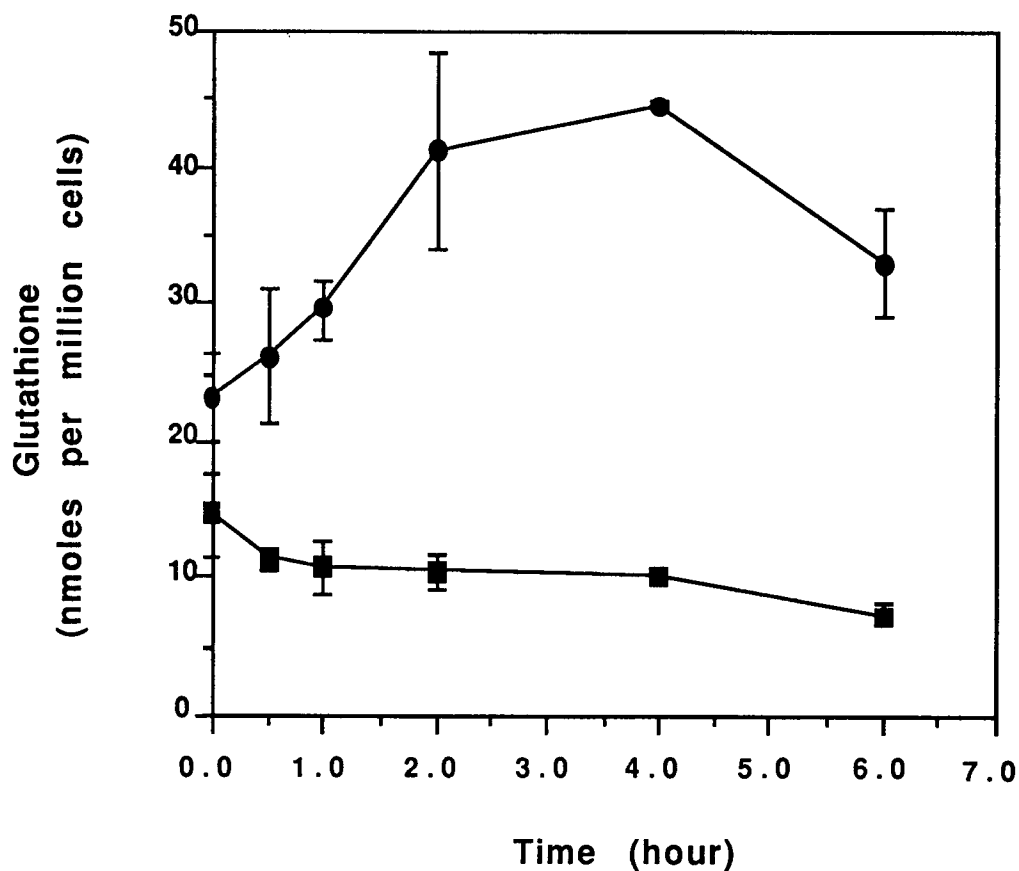


Figure 10. Modulation of Intracellular Glutathione in Isolated Rat Hepatocytes. Intracellular modulation of glutathione was accomplished by modifying the conditions of hepatocyte isolation and incubation. The effect on intracellular glutathione of isolation and incubation of hepatocytes with media lacking sulfur amino acids (squares) was markedly different from isolation and incubation of hepatocytes in media containing sulfur amino acids (circles). The data represent at least three separate experiments \pm SE.

Figure 11. 1,2-Dibromoethane-induced Lactate Dehydrogenase Leakage from Isolated Rat Hepatocytes. Panel "A" represents lactate dehydrogenase leakage from hepatocytes isolated/incubated in the absence of sulfur amino acids (system-1) whereas panel "B" represents isolation/incubation in the presence of sulfur amino acids (system-2). The data represent at least 3 separate experiments \pm SE. "*" denotes that this value is significantly different from control at this and subsequent time points ($P \leq 0.01$). DBE = 1,2-dibromoethane.

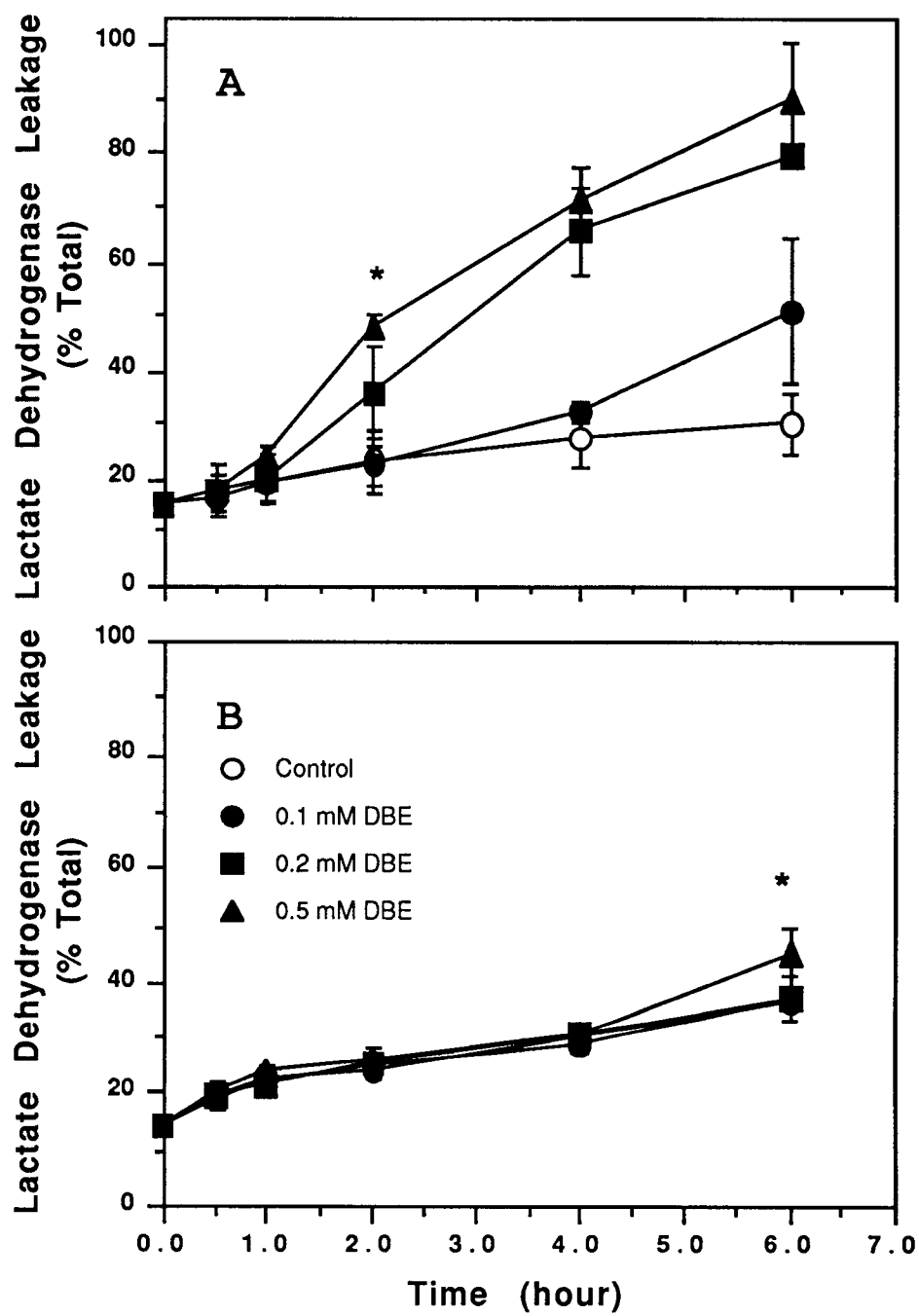


Figure 11.

Figure 12. 1,2-Dibromoethane-induced Depletion of Intracellular Glutathione in Isolated Rat Hepatocytes. This figure depicts the 1,2-dibromoethane-induced depletion of intracellular glutathione in hepatocytes under conditions of isolation/incubation in the absence (system-1) (A) or presence (system-2) (B) of sulfur amino acids. The data represent at least 3 separate experiments \pm SE. "*" denotes that this determination is significantly different from control at this and subsequent time points ($P \leq 0.05$). "+" denotes that the 1,2-dibromoethane-induced depletions were to levels significantly different than control ($P \leq 0.01$). DBE = 1,2-dibromoethane.

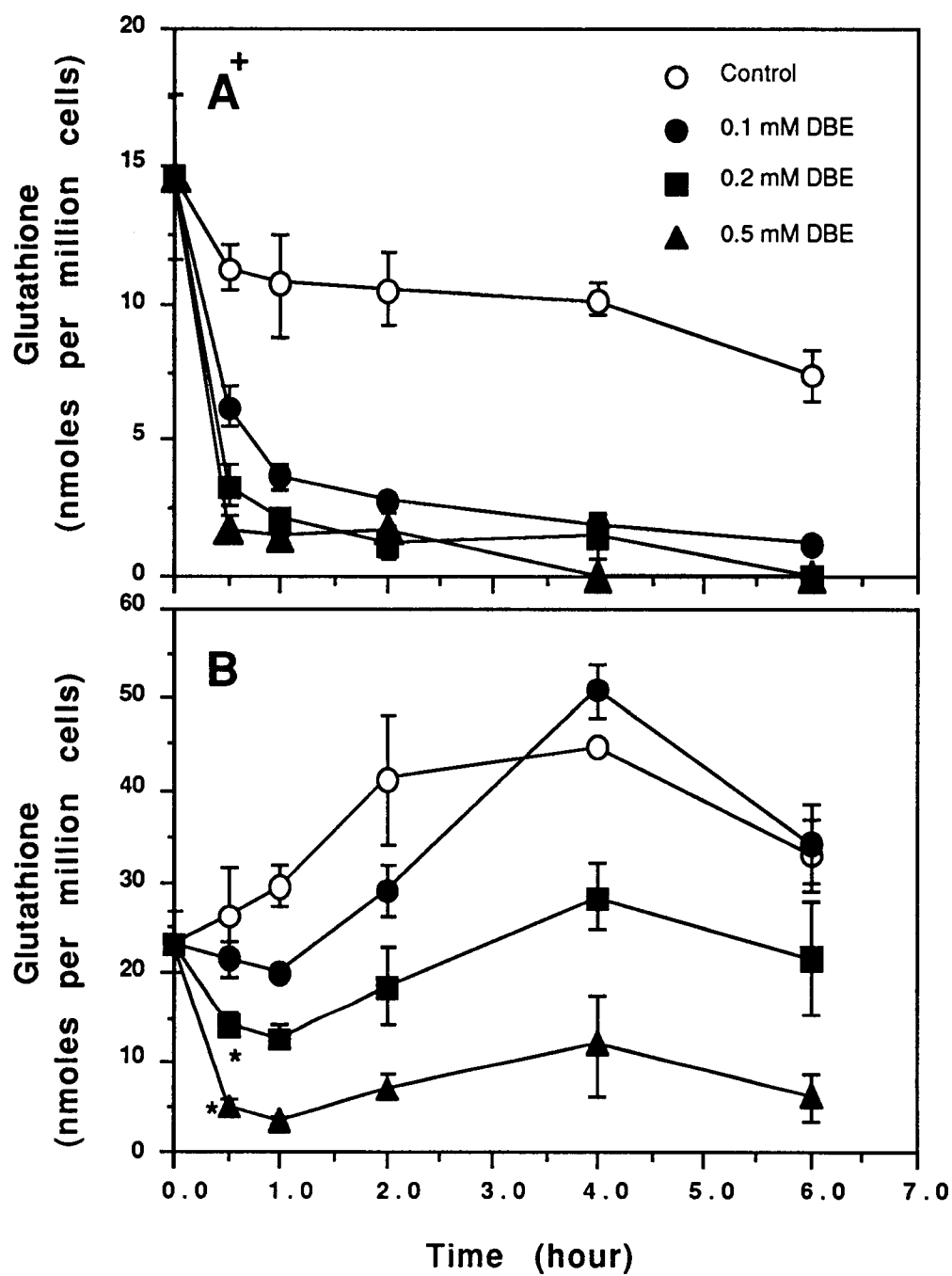


Figure 12.

levels (0.5 nmoles per million cells) for 500 and 200 μM 1,2-dibromoethane, respectively. In contrast, under system-2 conditions, glutathione depletion to less than 5 nmoles of glutathione per million cells occurred only after 500 μM 1,2-dibromoethane exposure. This took place within the first hour of incubation but did not persist as the level of intracellular glutathione increased to more than 7.0 nmoles per million cells by the second hour of incubation and remained above 5 nmoles per million cells for the remainder of the incubation period. 1,2-Dibromoethane (200 μM) did not deplete intracellular glutathione below 10 nmoles of glutathione per million cells at any time during the incubations. 1,2-dibromoethane (100 μM) exposure gave rise to a slight depletion of intracellular glutathione within the first hour of incubation followed by repletion to control levels by the fourth hour of incubation.

The formation of thiobarbituric acid-reactive species is indicative of lipid peroxidation. Thiobarbituric acid-reactive species formation occurred in a dose- and time-dependent manner following 1,2-dibromoethane exposure of hepatocytes in system-1 but not system-2 (Figure 13). The generation of thiobarbituric acid reactive species was concomitant with the leakage of lactate dehydrogenase.

The effect of various agents on 1,2-dibromoethane-induced cell injury was assessed. These experiments were performed with hepatocytes under system-1 conditions. The agents were added to the cell suspensions following the first hour of 1,2-dibromoethane treatment.

Butylated hydroxyanisole and desferal mesylate, two antioxidants with different mechanisms of action, were found to inhibit both lactate dehydrogenase leakage and thiobarbituric acid-reactive species formation as shown in figures 14 and 15, respectively.

Figure 13. 1,2-Dibromoethane-induced Formation of Thiobarbituric Acid-reactive Species in Isolated Rat Hepatocytes. This figure depicts the 1,2-dibromoethane-induced formation of thiobarbituric acid-reactive species in hepatocytes under conditions of isolation/incubation in the absence (A) or presence (B) of sulfur amino acids. The data represent at least 3 separate experiments \pm SE. "*" denotes that this determination is significantly different from control at this and subsequent time points ($P \leq 0.01$). DBE = 1,2-dibromoethane.

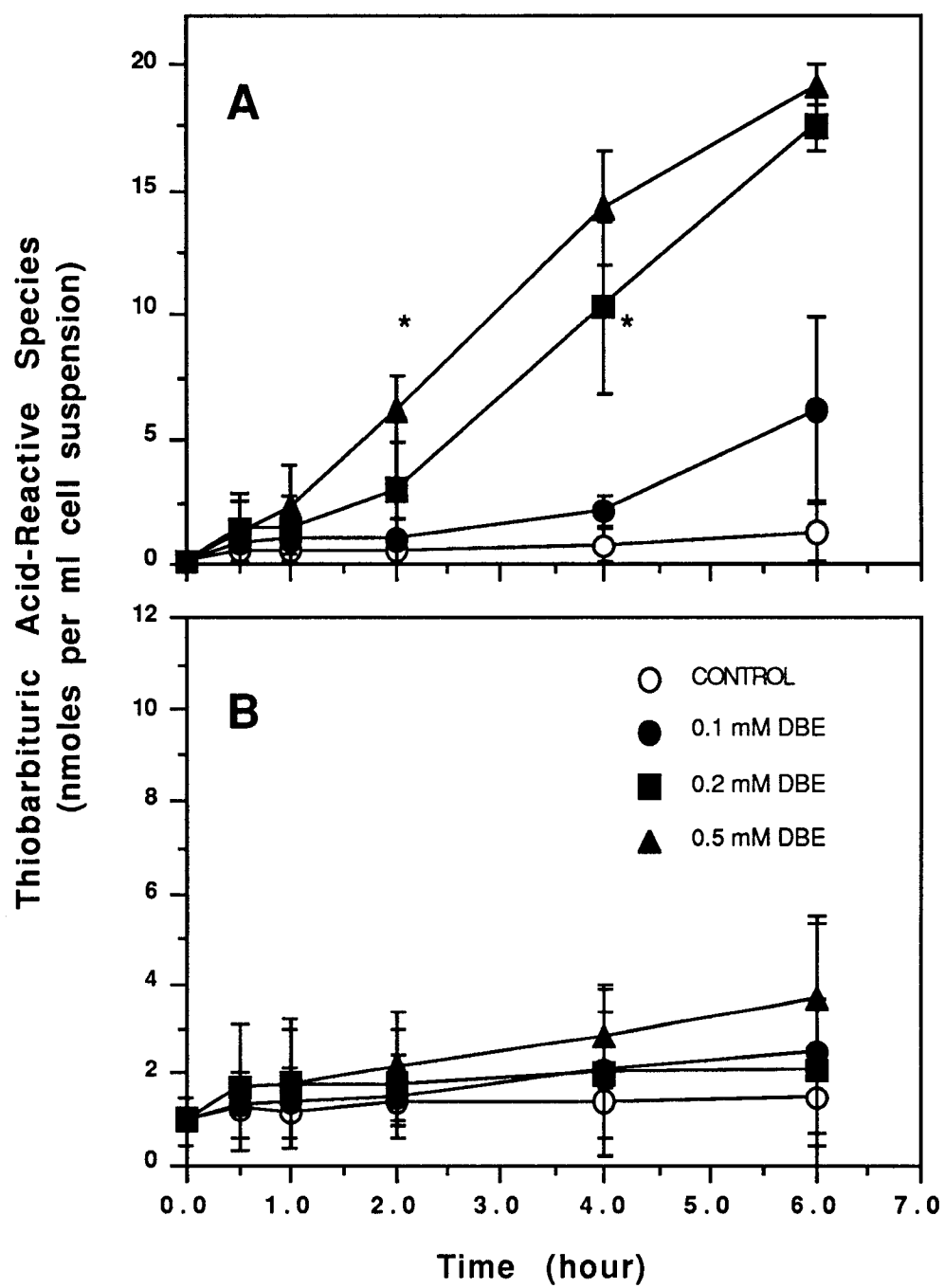


Figure 13.

Figure 14. The Effect of Butylated Hydroxyanisole on 1,2-Dibromoethane Toxicity in Isolated Rat Hepatocytes. Butylated hydroxyanisole (BHA) (0.1 mM) was added to the cell suspensions following 1 hour of 1,2-dibromoethane (DBE) exposure (0.5 mM). "*" denotes that this determination and all subsequent determinations are significantly different from the control and 1,2-dibromoethane-treated hepatocytes with butylated hydroxyanisole ($P \leq 0.05$). Thiobarbituric acid-reactive species (TBA-RS).

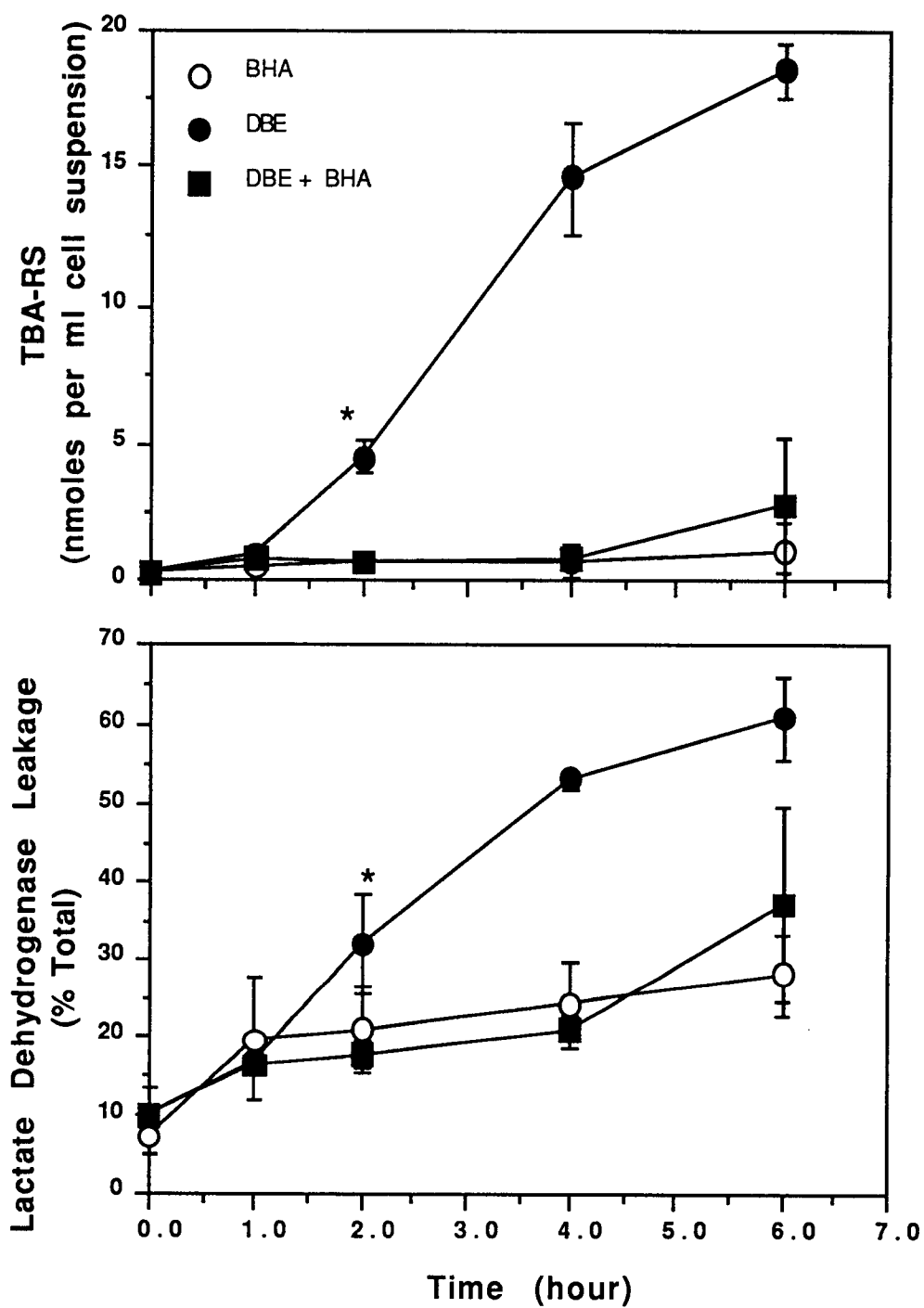


Figure 14.

Figure 15. The Effect of Desferal Mesylate on 1,2-Dibromoethane Toxicity in Isolated Rat Hepatocytes. Desferal mesylate (DESF) (0.2 mM) was added to the cell suspensions following 1 hour of 1,2-dibromoethane (DBE) (0.5 mM) exposure. "*" denotes that this determination and all subsequent determinations are significantly different from the control and 1,2-dibromoethane-treated hepatocytes with desferal mesylate ($P \leq 0.05$). Thiobarbituric acid-reactive species (TBA-RS).

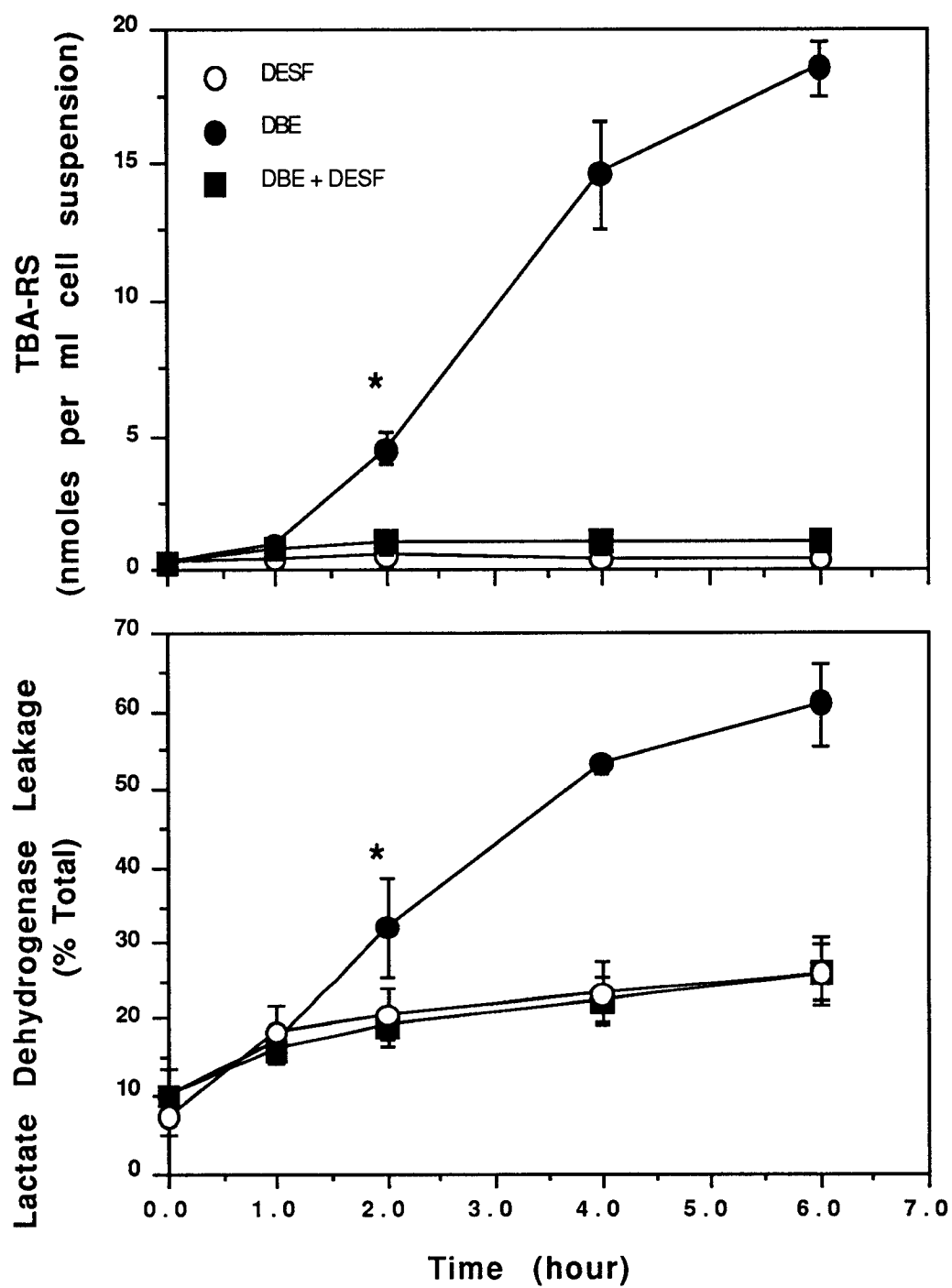


Figure 15.

N-Acetyl-cysteine, a source of free thiol and potential supplier of cysteine for glutathione biosynthesis, reduced the extent of lactate dehydrogenase leakage from hepatocytes without reducing thiobarbituric acid-reactive species formation (Figure 16). The leakage of lactate dehydrogenase from 1,2-dibromoethane-treated hepatocytes was reduced by 43, 40 and 10% at 2, 4 and 6 hours of incubation, respectively. Thiobarbituric acid-reactive species formation was not different from control through 4 hours of incubation and increased to 170% of control by 6 hours of incubation.

Ruthenium Red, an agent that influences the cellular transport of calcium, had no effect on lactate dehydrogenase leakage but was found to lower the thiobarbituric acid-reactive species formation at 4 and 6 hours of incubation (Figure 17). Ruthenium red inhibited the formation of thiobarbituric acid-reactive species by 39 and 38% at 4 and 6 hours of incubation, respectively.

Figure 16. The Effect of N-Acetyl-cysteine on 1,2-Dibromoethane Toxicity in Isolated Rat Hepatocytes. N-Acetyl-cysteine (N-acetyl-cys) (10.0 mM) was added to the cell suspensions following 1 hour of 1,2-dibromoethane (DBE) (0.5 mM) exposure. "*" denotes that this determination is significantly different from the N-acetyl-cysteine and 1,2-dibromoethane treated hepatocytes ($P \leq 0.01$). "+" denotes significantly different than control ($P \leq 0.01$). "#" denotes that this value is significantly different than control and 1,2-dibromoethane + N-acetyl-cysteine treated hepatocytes ($P \leq 0.05$). Thiobarbituric acid-reactive species (TBA-RS).

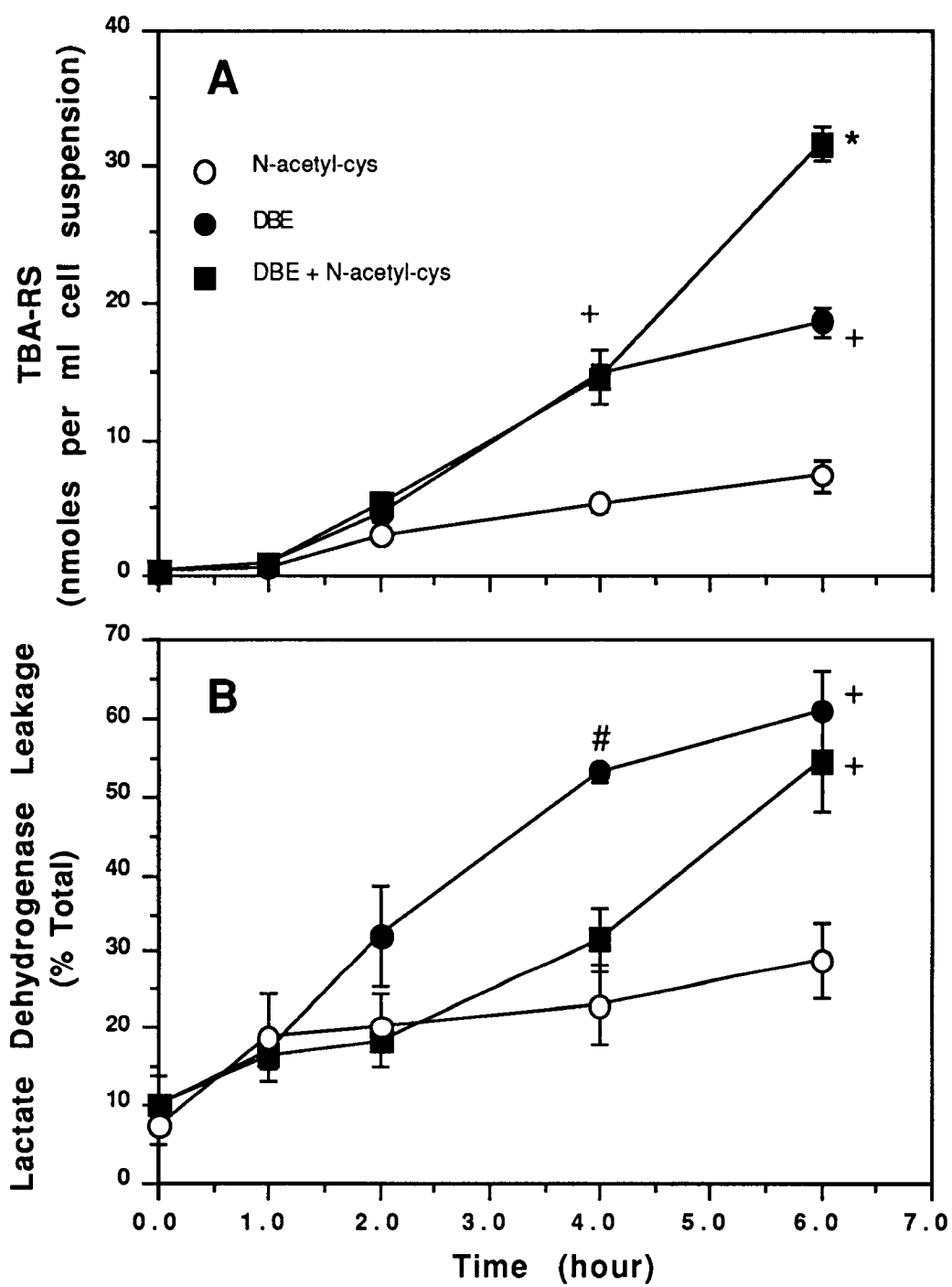


Figure 16.

Figure 17. The Effect of Ruthenium Red on 1,2-Dibromoethane Toxicity in Isolated Rat Hepatocytes. Ruthenium red (25.0 μ M) was added to the cell suspensions following 1 hour of 1,2-dibromoethane (DBE) (0.5 mM) exposure. "*" denotes that this and subsequent determinations are significantly different from the ruthenium red control ($P \leq 0.01$). "+" denotes significantly different than 1,2-dibromoethane treated hepatocytes ($P \leq 0.05$) at this and subsequent determinations. Thiobarbituric acid-reactive species (TBA-RS).

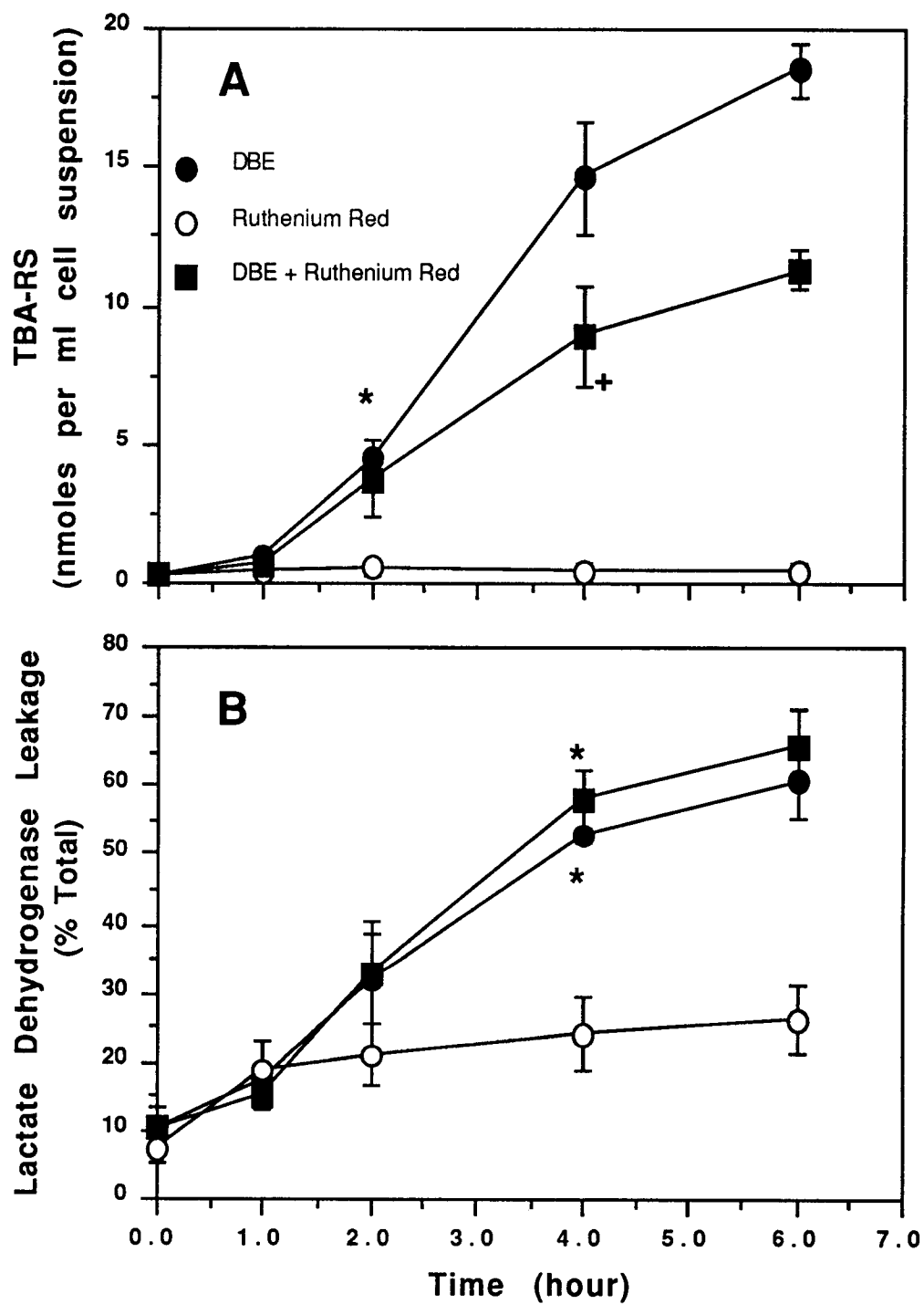


Figure 17.

Discussion

This investigation has demonstrated that maintenance of intracellular glutathione to levels above 5.0 nmoles per million cells protected against 1,2-dibromoethane-induced lipid peroxidation and cell death. Freshly isolated hepatocytes were shown in the previous chapter to utilize glutathione extensively in 1,2-dihaloethane metabolism. 1,2-Dihaloethane induced glutathione depletion and the formation of at least three glutathione-containing metabolites. The formation of a reactive sulfur half-mustard, S-(2-haloethyl)glutathione, was thought responsible for most of the glutathione depletion, metabolite formation, and covalent binding of 1,2-dibromoethane to hepatocyte protein.

S-(2-Chloroethyl)glutathione, a representative glutathione sulfur half-mustard, was shown to be an active alkylating agent toward a variety of model compounds including glutathione (Jean and Reed, 1989). Therefore, the maintenance of an adequate amount of intracellular glutathione may have afforded protection against 1,2-dibromoethane-derived glutathione sulfur half-mustard alkylation of cellular macromolecules. However, 1,2-dibromoethane metabolism may give rise to reactive metabolites other than the glutathione sulfur half-mustard. Increased intracellular glutathione may have provided protection against these metabolites as well.

The depletion of intracellular glutathione by 1,2-dihaloethanes may itself result in the generation of injurious oxidative stress. Such a situation could arise by virtue of reducing the cell's capacity to deal adequately with the endogenous generation of reactive oxygen species. It has been estimated that 2-5% of the mitochondrial

consumption of oxygen results in the formation of hydrogen peroxide (Chance et al., 1979). Glutathione and the glutathione redox cycle represent a major cellular defense system against hydrogen peroxide as well as other organic peroxides (Reed, 1986). Glutathione depletion to levels below 10-20% of normal may severely reduce the cell's ability to defend against oxidative injury (Younges and Siegers, 1981).

1,2-Dibromoethane-induced glutathione depletion preceded the formation of thiobarbituric acid-reactive species, an indicator of lipid peroxidation. In agreement with the above proposal, the maintenance of intracellular glutathione at levels above 5 nmoles per million cells (approximately 14% of normal, assuming a normal glutathione content of 35 nmoles per million cells) was associated with decreased lipid peroxidation. In addition, the maintenance of intracellular glutathione inhibited lactate dehydrogenase leakage, ie. cell death.

Cell death and lipid peroxidation occurred concomitantly in 1,2-dibromoethane-treated hepatocytes following glutathione depletion. This observation led to the investigation of the role of lipid peroxidation in 1,2-dibromoethane-induced cell death.

The antioxidant, butylated hydroxyanisole, and the iron chelator, desferal mesylate, inhibited both lipid peroxidation and cell death. An observation suggesting that lipid peroxidation may be principally involved in 1,2-dibromoethane-induced cell death. The effect of desferal mesylate would indicate the importance of iron in the lipid peroxidation process.

N-Acetyl-cysteine (10 mM) reduced lactate dehydrogenase leakage through 4 hours of incubation without a reduction in lipid peroxidation. This effect was not

related to increased intracellular glutathione as N-acetylcysteine did not promote glutathione synthesis. Therefore its effects were most likely related to providing the cells with additional free thiol. The additional free thiol may have protected critical protein thiols from modification by reactive 1,2-dibromoethane metabolites or products of lipid peroxidation.

Ruthenium red (25 μ M) did not protect against 1,2-dibromoethane cell death but did reduce the amount of lipid peroxidation. This compound has been shown to inhibit the uptake of calcium by isolated mitochondria (Reed and Bygrave, 1974), to reduce plasma membrane Ca-ATPase activity (Missiaen *et al.*, 1990) and inhibit the release of calcium from sarcoplasmic reticulum (Kanmura *et al.*, 1989). The results with ruthenium red would suggest that calcium fluxes responsive to ruthenium red modulation are not involved to any great extent in 1,2-dibromoethane-induced toxicity in isolated rat hepatocytes under these experimental conditions.

The effect of these various agents on lipid peroxidation and cell death suggest that lipid peroxidation can play a significant role in the development of 1,2-dibromoethane-induced cell death in this model system. Whether lipid peroxidation is due to extensive glutathione depletion or the result of other cellular injury involving the interaction of 1,2-dibromoethane reactive metabolites with other cellular constituents is not known.

ALPHA-NAPHTHYLISOTHIOCYANATE TOXICITY AND GLUTATHIONE DEPLETION IN FRESHLY ISOLATED RAT HEPATOCYTES.

Introduction

Cholestasis may occur in humans as a side effect of specific drugs or viral infections. This dysfunction may result from a variety of circumstances ranging from the intra- and extrahepatic obstruction of bile ducts to the inhibition of bile acid formation within hepatocytes. Our understanding of drug-induced cholestasis in humans is very incomplete due to the complex nature of bile formation, bile flow, and the infrequency of drug-induced cholestasis in humans.

alpha-Naphthylisothiocyanate has been used extensively to induce intrahepatic cholestasis in animals as a model system for study. alpha-Naphthylisothiocyanate-induced cholestasis is associated with a variety of effects including hyperbilirubinemia, serum elevations of bile acids and marker enzymes indicative of liver injury, inflammation of portal tracts, necrosis of interlobular bile ducts and focal necrosis of hepatocytes (Plaa and Priestly, 1977). The mechanism(s) responsible for these effects is unknown. However, metabolism of alpha-naphthylisothiocyanate to a toxic species by the mixed-function oxidase system has been suggested (Roberts and Plaa, 1965).

Induction of mixed-function oxidase activity was shown to enhance alpha-naphthylisothiocyanate liver injury whereas the inhibition of mixed-function oxidase activity diminished alpha-naphthylisothiocyanate liver injury (Roberts and Plaa, 1965). These observations only offer an indirect

association linking mixed-function oxidase activity to alpha-naphthylisothiocyanate toxicity. A toxic alpha-naphthylisothiocyanate metabolite has yet to be identified or its mechanism of action defined. Therefore, it is altogether possible that mixed-function oxidase metabolism may have little to do with alpha-naphthylisothiocyanate-induced liver injury.

The agents used to modulate mixed-function oxidase activity are also known to influence the activity of other enzyme systems. Recently, investigators (Dahm and Roth, 1991) have recognized that the agents used to induce mixed-function oxidase activity have been shown to increase hepatic glutathione content and glutathione transferase activity. In addition, the inhibitors of mixed-function oxidase activity are known to decrease hepatic glutathione content and glutathione transferase activity. The modulation of glutathione and glutathione transferase activity may have contributed to the observed potentiation and/or inhibition of alpha-naphthylisothiocyanate toxicity. It is interesting that the effects of the "modulating" agents suggest that glutathione and glutathione transferase function to promote alpha-naphthylisothiocyanate toxicity.

Dahm and Roth (1991) recently reported that modulation of hepatic nonprotein thiol (presumably glutathione) greatly influenced alpha-naphthylisothiocyanate liver injury. They observed that depletion of hepatic nonprotein thiol with buthionine sulfoximine, diethyl maleate or phorone protected against alpha-naphthylisothiocyanate-induced liver injury. In addition, the repletion of hepatic nonprotein thiol content was coincident with the development of alpha-naphthylisothiocyanate liver injury. These observations lend support to the hypothesis that nonprotein thiols are involved in the development of alpha-naphthylisothiocyanate-

induced liver injury.

In an effort to better define the role of glutathione in alpha-naphthylisothiocyanate-induced liver injury its effect on intracellular glutathione and toxicity were examined in freshly isolated rat hepatocytes. The spontaneous, nonenzymatic reaction of alpha-naphthylisothiocyanate with glutathione was also studied. The results presented herein have recently been presented elsewhere (Carpenter-Deyo *et al.*, 1991).

Materials and Methods

Chemicals:

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Solvents were HPLC grade (OmniSolv, EM Science, Gibbstown, NJ) except dimethylsulfoxide (DMSO) (AR grade, Mallinckrodt Inc., Paris, KT) and deuterated NMR solvents which were purchased from Aldrich Chemical Company (Milwaukee, WI).

Synthesis of Glutathione-alpha-naphthylisothiocyanate conjugate:

30 mg of alpha-naphthylisothiocyanate (0.16 mM) was dissolved in 1.0 ml of acetonitrile. 350 mg of glutathione dissolved in 4.0 ml of 0.10 M TRIS buffer, pH = 7.0, was added dropwise to the solution of alpha-naphthylisothiocyanate over a 2 minute period. After stirring at room temperature for 5 minutes, the pH was adjusted to < 4 with the addition of 1.0 ml glacial acetic acid. The glutathione conjugate was then purified via high-pressure liquid chromatography (HPLC) utilizing a 9.6 mm x 300 mm reverse-phase C18 column (Custom LC, Houston, TX). A mobile phase of 20% acetonitrile, 0.1% acetic acid was used to elute the material from the column into a collection flask immersed in liquid nitrogen. The mobile phase was removed by lyophilization. Post column detection at 308 nm allowed detection of the glutathione conjugate. The glutathione conjugate was stored at -80°C after lyophilization.

The identity of the glutathione-alpha-naphthylisothiocyanate conjugate was verified by Fast Atom Bombardment Mass Spectroscopy (FAB-MS) and thin layer chromatography (TLC). FAB-MS analysis was performed with a

Kratos MS50TC RF mass spectrometer, a 7 KV xenon beam for ionization and a glycerol matrix. TLC analysis involved spotting sample onto silica gel TLC plates (Analtech, Newark, DE) and development with isopropyl alcohol/water/acetic acid (85/14/1). Rf value comparisons were made between glutathione, alpha-naphthylisothiocyanate and the glutathione-alpha-naphthylisothiocyanate conjugate as observed with UV light or ninhydrin reaction. Proton magnetic resonance analysis was attempted with little success due to the conjugates instability and low solubility in aqueous solutions. However, the spectra obtained did give an indication that both alpha-naphthylisothiocyanate and glutathione were present in the sample.

Isolation and Treatment of Hepatocytes:

Hepatocytes were isolated from male Sprague-Dawley rats (180-200 gm) according to Fariss and Reed (1987) and suspended (2.0×10^6 cells per ml) in a modified Fischer's media (lacking sulfur amino acids) containing 2.5 mM calcium and 10 mM 4-((2-hydroxyethyl)-piperazine)ethanesulfonic acid. Initial viability was greater than 90% as assessed by trypan blue exclusion.

Aliquots of cell suspension (15 ml) were treated with vehicle (DMSO, 100 μ l), alpha-naphthylisothiocyanate, or the glutathione-alpha-naphthylisothiocyanate conjugate. Cells were then incubated at 37°C under an atmosphere of 95% O₂, 5% CO₂ for up to 4 hours. Aliquots of cell suspension were taken prior to and at various times after addition of agents to assess lactate dehydrogenase leakage, thiobarbituric acid-reactive species formation, intra- and extracellular glutathione content.

To determine if isolated hepatocytes produce and excrete the glutathione-alpha-naphthylisothiocyanate

conjugate, aliquots of extracellular media were analyzed following exposure. Hepatocytes, isolated as above were resuspended to 5.0×10^6 cells per ml in the modified Fischer's media were treated with 1.0 mM alpha-naphthylisothiocyanate. At various times 1.0 ml aliquots were taken, centrifuged at 12,000 rpm to pellet the cells and 0.5 ml of media immediately frozen with liquid nitrogen. These samples were lyophilized and subjected to HPLC analysis to test for the presence of glutathione-alpha-naphthylisothiocyanate conjugate.

HPLC Analysis:

HPLC analysis involved dissolving the lyophilized material in 0.2 ml of 35% acetonitrile containing either 1.0 M sodium acetate (pH = 4.0) or 0.05 M sodium phosphate (pH = 8.0), and centrifugation to remove insoluble material prior to HPLC analysis. Aliquots of sample were injected onto a reverse phase C18 column (Custom LC, Houston, TX) 4.6 mm X 250 mm. The elution of material from the column was accomplished by linearly increasing the content of acetonitrile in 0.05 M sodium acetate (pH = 4.0) from 10% to 70% over a 15 minute period. Post column detection was made at 308 nm with a SpectroFlow 757 detector.

Biochemical Analysis:

The separation of viable cells from non-viable cells was achieved as described by Fariss et al. (1985) and the quantification of extra- and intracellular glutathione and glutathione disulfide as described by Fariss and Reed (1987). Lactate dehydrogenase leakage from the hepatocytes was determined spectrophotometrically as described by Fariss et al. (1985) with the use of a Kontron spectrophotometer (UVikon 810). Thiobarbituric acid-reactive species

formation was performed as described by Stacey and Klaassen (1981).

Statistical Analysis:

Data were analyzed using a one-way analysis of variance (ANOVA) program from Statview (Brainpower, INC., Calabasas, CA) and means were compared by a Dunnett's test. $P \leq 0.05$ was chosen as the level of significance.

Results

Freshly isolated hepatocytes were incubated with 0, 50 100 and 500 μM alpha-naphthylisothiocyanate for up to 4 hours. The leakage of lactate dehydrogenase from the cells was used to assess toxicity. Lactate dehydrogenase leakage did not differ from controls except for 500 μM alpha-naphthylisothiocyanate exposure as shown in figure 18. The increase in lactate dehydrogenase leakage began after 2 hours of incubation and increased to almost 100% by 4 hours.

Glutathione depletion was observed for all of the alpha-naphthylisothiocyanate exposures but varied in the extent and time of depletion (Figure 19). alpha-Naphthylisothiocyanate (50 μM) decreased intracellular glutathione to 60% of control by 1 hour of incubation followed by total repletion by 4.0 hours. alpha-Naphthylisothiocyanate (100 μM) decreased intracellular glutathione to 35% of control by 1.0 hour and remained at this level for the rest of the incubation. alpha-Naphthylisothiocyanate (500 μM) mimicked the 100 μM treatment for the first 2.0 hours of incubation and then the intracellular glutathione content dropped to levels below the limit of detection (0.5 nmoles per million cells) by 4 hours.

The alpha-naphthylisothiocyanate-induced loss of intracellular glutathione was concomitant with the appearance of extracellular glutathione and glutathione disulfide. Figure 20 depicts the time-dependent accumulation of glutathione equivalents in the extracellular medium. Unlike the depletion of intracellular glutathione the appearance of glutathione equivalences in the extracellular medium showed only a slight dependence on dose. Each of the exposures caused the accumulation of

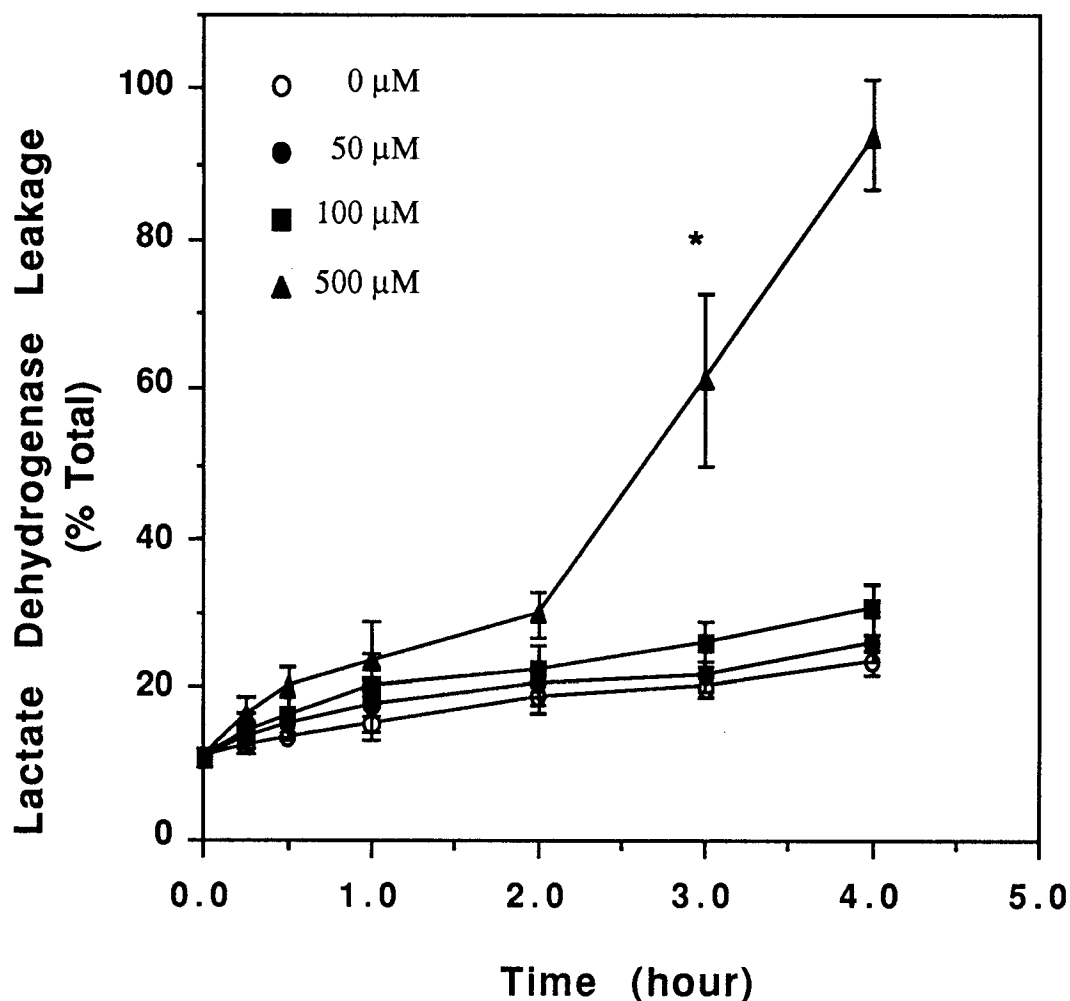


Figure 18. α -Naphthylisothiocyanate-induced Release of Lactate Dehydrogenase from Isolated Rat Hepatocytes. Hepatocytes in suspension were treated with 0, 50, 100 and 500 μ M α -naphthylisothiocyanate (dimethylsulfoxide as vehicle) for up to four hours and analyzed as described in the "Materials and Methods" section. Lactate dehydrogenase leakage is expressed as the % of total lactate dehydrogenase releasable by 0.4 % Triton X-100 at time zero. Data represent the mean \pm SE of at least three separate experiments. (*) denotes significantly different from control ($p < 0.05$).

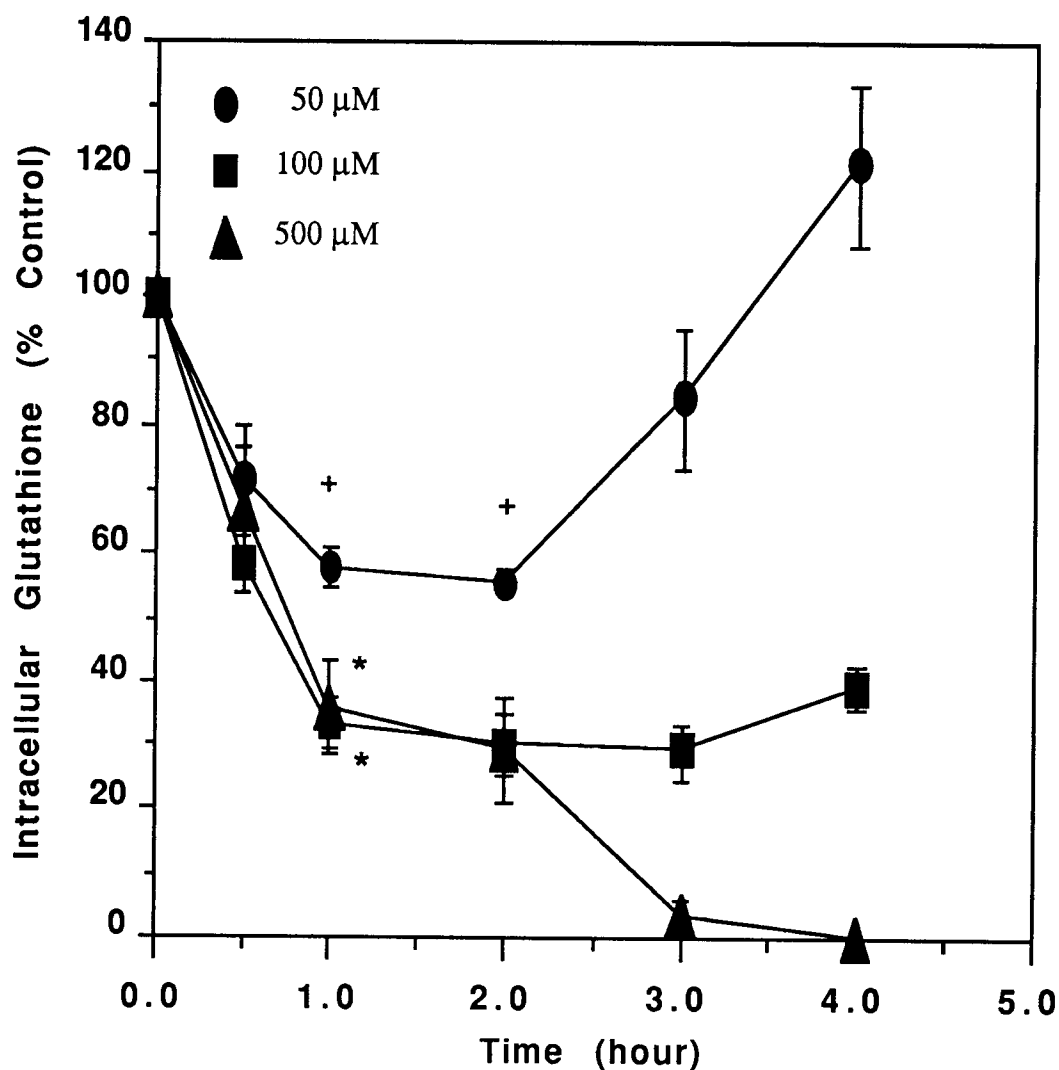


Figure 19. α -Naphthylisothiocyanate-induced Depletion of Intracellular Glutathione from Isolated Rat Hepatocytes. Hepatocytes in suspension were treated with 0, 50, 100 and 500 μ M α -naphthylisothiocyanate (dimethylsulfoxide as vehicle) for up to four hours and analyzed as described in the "Materials and Methods" section. Intracellular glutathione is expressed as % of the control for that time point. Data represent the mean \pm SE of at least three separate experiments. (*) denotes significantly different from control at this time point and all subsequent time points ($p < 0.05$). (+) denotes significantly different from control at this time point.

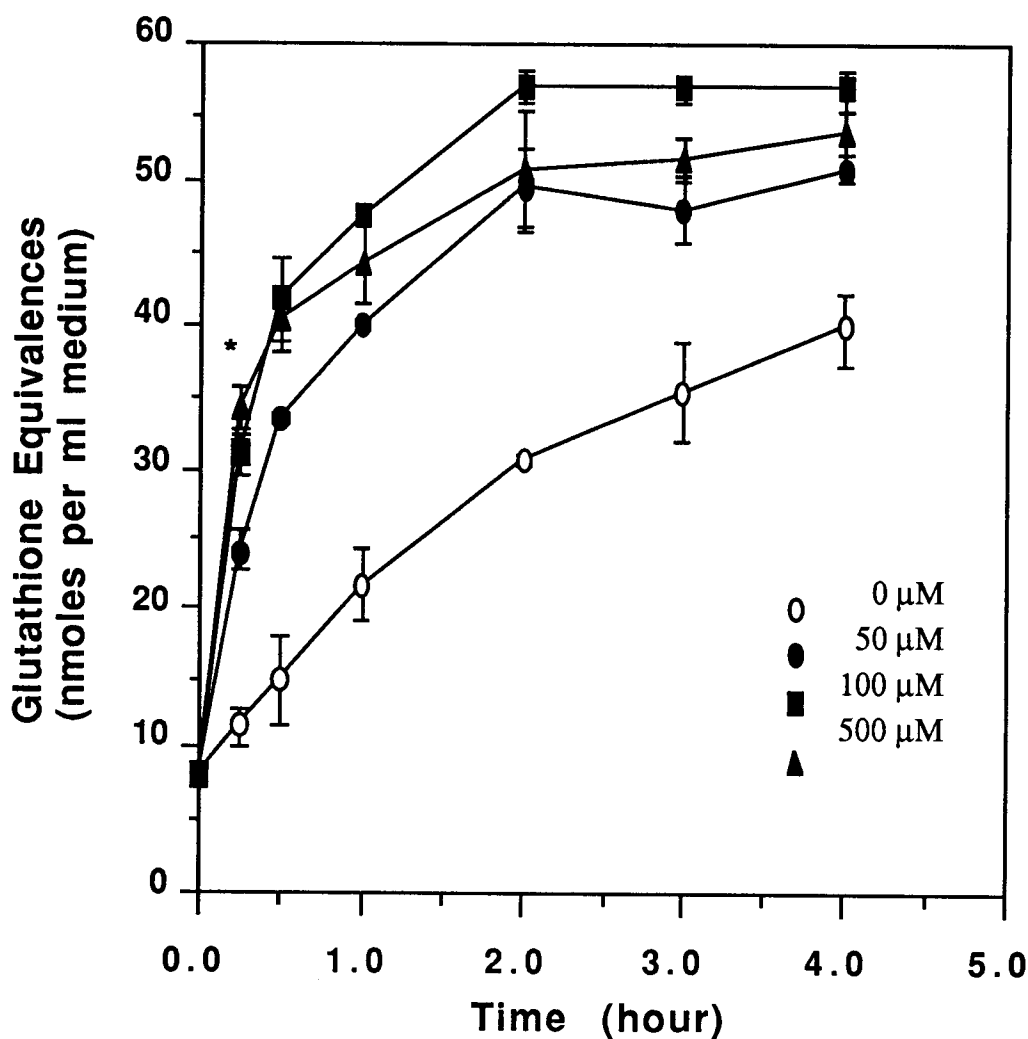


Figure 20. α -Naphthylisothiocyanate-induced Extracellular Accumulation of Glutathione. Hepatocytes in suspension were treated with 0, 50, 100 and 500 μ M α -naphthylisothiocyanate (dimethylsulfoxide as vehicle) for up to four hours and analyzed as described in the "Materials and Methods" section. Values represent the sum total of glutathione as the reduced form and that amount present as glutathione disulfide. Data represent the mean \pm SE of at least three separate experiments. (*) denotes that treatment with α -naphthylisothiocyanate resulted in the extracellular accumulation of glutathione to levels significantly greater than the control ($p < 0.05$).

extracellular glutathione to approximately 50-55 μM in 2 hours with no significant changes thereafter.

The interaction of alpha-naphthylisothiocyanate with glutathione was assessed via HPLC. HPLC analysis of alpha-naphthylisothiocyanate in 35% acetonitrile, 50 mM potassium phosphate buffer, pH = 7.5 gave rise to a single peak in the chromatogram representing alpha-naphthylisothiocyanate. The addition of glutathione to this solution resulted in the reduction in size of the alpha-naphthylisothiocyanate peak and the appearance of new peak in the chromatogram. This new peak elutes from the reverse phase column well after glutathione but before alpha-naphthylisothiocyanate. The simplicity of the reaction mixture and the retention time characteristics suggest that this new peak represents the formation of a glutathione-alpha-naphthylisothiocyanate conjugate. FAB-MS analysis of this material supports the contention that this material is a glutathione-alpha-naphthylisothiocyanate conjugate (Figure 21). In addition, the mass spectrum contains evidence that the material is a glutathione S-conjugate. This glutathione S-conjugate was further characterized by Dr. Dan Marchand and found to be stable in acid and unstable in neutral or alkaline solutions.

The presence of the glutathione-alpha-naphthylisothiocyanate conjugate in the extracellular media of alpha-naphthylisothiocyanate treated cells was assessed via HPLC. Aliquots of cell suspension were taken at various times following exposure to alpha-naphthylisothiocyanate. HPLC analysis of the cell-free medium indicated that the glutathione S-conjugate was present (Figure 22). The HPLC chromatogram of medium from exposed cells contained four distinct peaks not present in control cells. One of these peaks coelutes with the purified glutathione-alpha-

Figure 21. Fast Atom Bombardment Mass Spectrum of the Glutathione- α -naphthylisothiocyanate Conjugate. The conjugate was dissolved into a glycerol matrix just prior to analysis as described in the "Materials and Methods" section. The molecular ion peak at m/Z 491 corresponds to that predicted for the molecular ion of the glutathione S-conjugate of α -naphthylisothiocyanate.

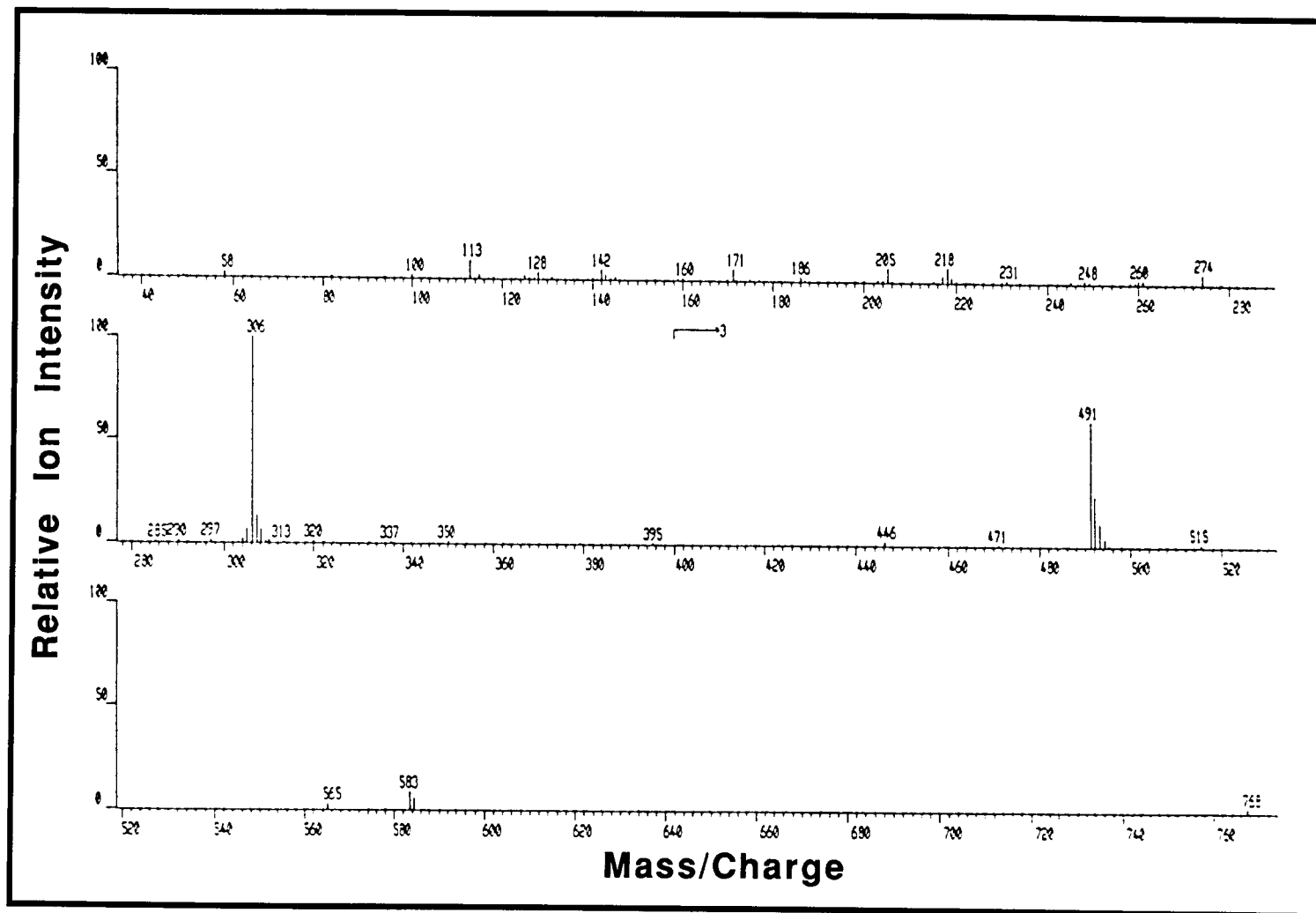


Figure 21.

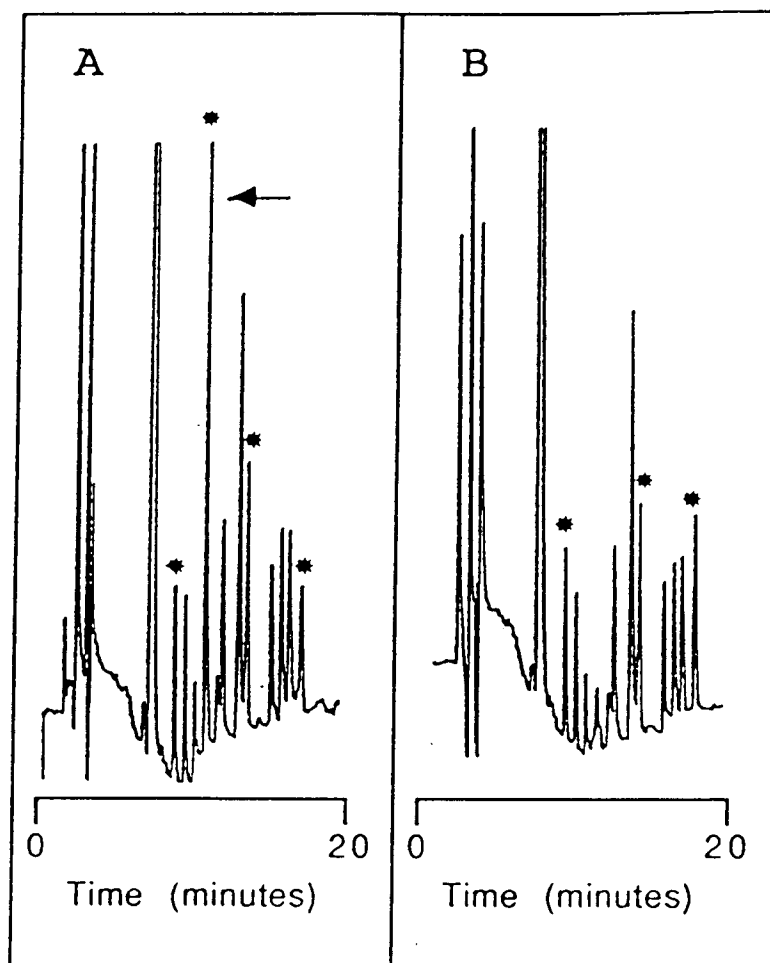


Figure 22. HPLC Analysis for the Presence of Glutathione S-conjugate of alpha-Naphthylisothiocyanate in Suspensions of Treated Hepatocytes. Hepatocytes (5.0×10^6 cells per ml) were treated with 1.0 mM alpha-naphthylisothiocyanate for two hours and aliquots taken and analyzed at various times as described in the "materials and "Methods" section. Sample preparation maximizing the stability of the glutathione S-conjugate allowed for its detection (chromatogram "A", arrow indicates peak assigned to the conjugate) whereas sample preparation known to destroy the conjugate resulted in disappearance of this peak (chromatogram "B"). The asterisks denote peaks not present in controls.

naphthylisothiocyanate conjugate and exhibits the same spectral characteristics and alkaline instability. These data suggest that this peak represents the same glutathione-alpha-naphthylisothiocyanate conjugate identified in the simple reaction mixture.

Freshly isolated hepatocytes were treated with 0, 100 and 500 μM glutathione-alpha-naphthylisothiocyanate conjugate for 4 hours just as was done for alpha-naphthylisothiocyanate. The effects on intracellular glutathione and leakage of lactate dehydrogenase are depicted in figures 23 and 24. The depletion of intracellular glutathione and lactate dehydrogenase leakage were almost identical to that observed for exposure to the same concentrations of alpha-naphthylisothiocyanate.

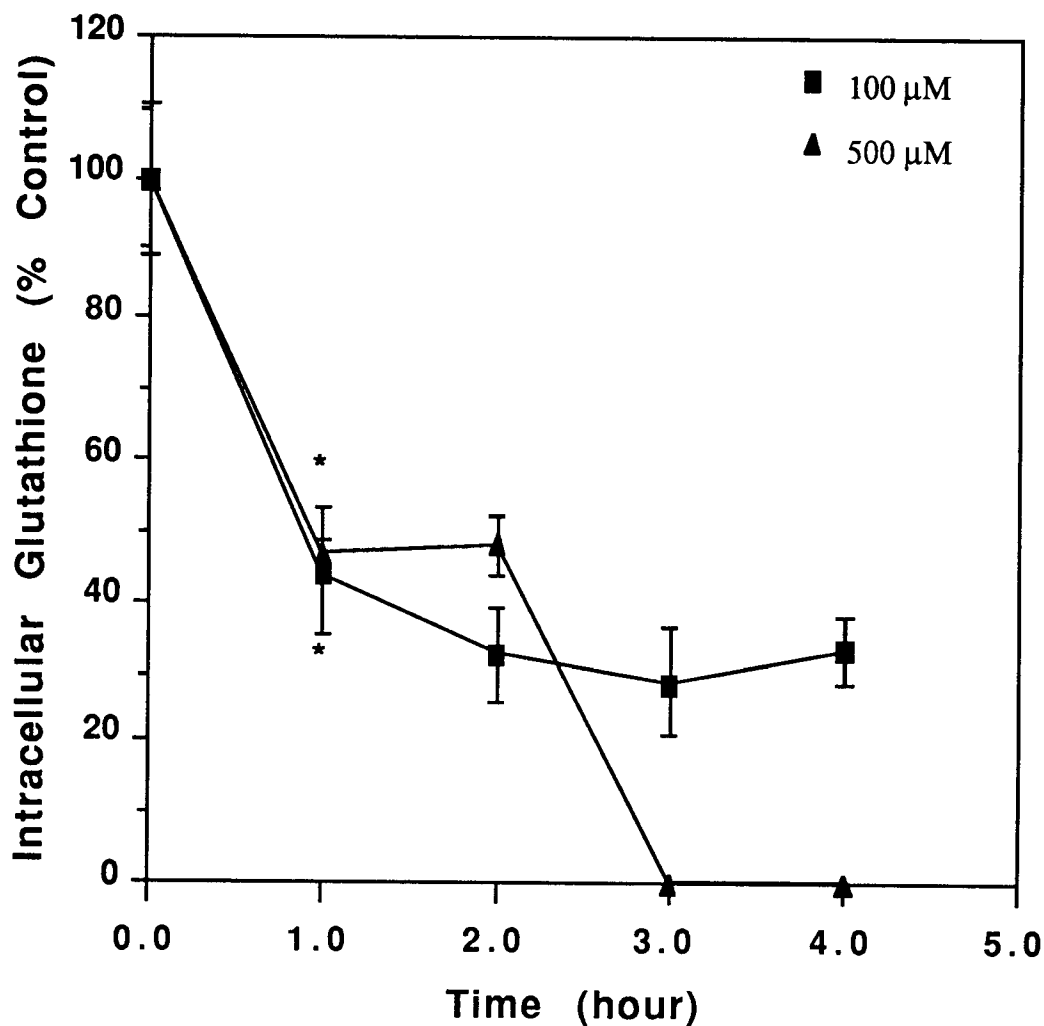


Figure 23. Effect of the Glutathione S-conjugate of alpha-Naphthylisothiocyanate on Intracellular Glutathione in Isolated Rat Hepatocytes. Hepatocytes were treated with the glutathione S-conjugate of alpha-naphthylisothiocyanate (0, 100 or 500 μ M) and analyzed as described in the "Materials and Methods" section. The data represent the mean \pm SE for at least three separate experiments. (*) denote significantly different than control at this time point and all subsequent time points ($p < 0.05$).

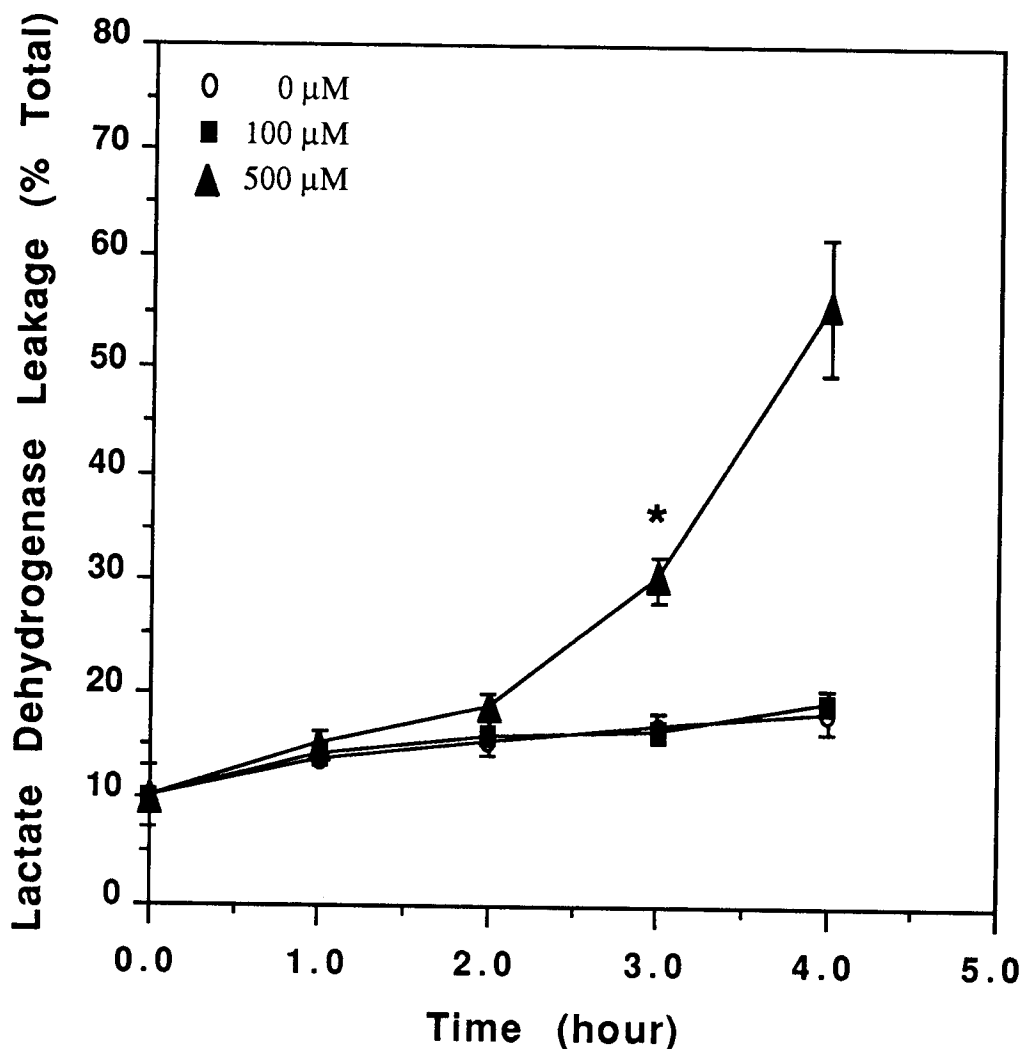


Figure 24. Effect of the Glutathione S-conjugate of alpha-Naphthylisothiocyanate on the Leakage of Lactate Dehydrogenase from Isolated Rat Hepatocytes. Hepatocytes were treated with the glutathione S-conjugate of alpha-naphthylisothiocyanate (0, 100 or 500 μM) and analyzed as described in the "Materials and Methods" section. The data represent the mean \pm SE for at least three separate experiments. (*) denote significantly different than control at this time point and all subsequent time points ($p < 0.05$).

Discussion

This investigation has demonstrated that exposure of freshly isolated hepatocytes to alpha-naphthylisothiocyanate produces a dose- and time-dependent depletion of intracellular glutathione. That 500 μ M alpha-naphthylisothiocyanate was toxic to the cells within 4 hours of exposure and that toxicity was associated with the depletion of glutathione to levels below 30% of control. Alpha-naphthylisothiocyanate was found to react with glutathione to form a glutathione S-conjugate. This conjugation was reversible and the conjugate found to be unstable in neutral solutions. The presence of this glutathione S-conjugate was observed in cell suspensions following treatment with alpha-naphthylisothiocyanate. The glutathione S-conjugate exhibited the same dose- and time-dependent depletion of intracellular glutathione and cellular toxicity as observed for alpha-naphthylisothiocyanate.

The reaction of alpha-naphthylisothiocyanate with glutathione was not unexpected. As a functional group, the isothiocyanates are known to be very reactive with nucleophiles, particularly sulfhydryls and amines. Dithiocarbamates, the product of sulfhydryl conjugation with isothiocyanates, have been shown to be unstable and dissociate to give back the thiol and isothiocyanate (Drobnica *et al.*, 1977). Studies with benzyl isothiocyanate have shown that its conjugation with glutathione may be spontaneous or catalyzed by the glutathione transferases (Brusewitz *et al.*, 1977). The conjugation of alpha-naphthylisothiocyanate with glutathione and the instability of the conjugate are characteristic of both isothiocyanates and their conjugates (Drobnica *et al.*, 1977).

The depletion of intracellular glutathione following exposure of isolated hepatocytes to alpha-naphthylisothiocyanate and the appearance of glutathione in the extracellular medium may best be explained as the result of a cyclic process. This process would involve passive diffusion of alpha-naphthylisothiocyanate into the cells, conjugation with glutathione, excretion of the glutathione S-conjugate into the medium via specific transport processes for glutathione conjugates, dissociation of the conjugate giving glutathione and alpha-naphthylisothiocyanate and passive diffusion of alpha-naphthylisothiocyanate back into the cell to begin another cycle. In this manner intracellular glutathione can be transported into the extracellular medium. The chemical characteristics of isothiocyanates and their conjugates as well as the presence of glutathione transport processes are in support of such a cycle.

According to this proposal, the cycling of alpha-naphthylisothiocyanate should lead to the total depletion of intracellular glutathione. However, total depletion of intracellular glutathione did not occur except for the highest dose of alpha-naphthylisothiocyanate. Depletion was associated with the loss of plasma membrane integrity, as indicated by the increased lactate dehydrogenase leakage.

One possible explanation for a limit on glutathione depletion relates to the accumulation of glutathione in the medium. The accumulation of glutathione or glutathione conjugates in the medium could potentially inhibit further excretion of glutathione conjugates, once a critical extracellular concentration is reached. This would appear to be approximately 50-55 μM . The concentration reached by all three of the alpha-naphthylisothiocyanate treatments.

There is also the possibility that metabolism of alpha-

naphthylisothiocyanate to a metabolite that no longer participates in the cycle may contribute to the limited accumulation of glutathione in the medium. The metabolism of alpha-naphthylisothiocyanate by liver microsomes has been demonstrated (Roberts, 1973). In addition, HPLC analysis of the media from treated cells revealed the presence of material not present in the control media. The unidentified material exhibited absorbance characteristics associated with alpha-naphthylisothiocyanate and are most likely metabolites of alpha-naphthylisothiocyanate that do not participate in the proposed cycle.

Toxicity, as assessed by the leakage of lactate dehydrogenase from hepatocytes, developed only after exposure to 500 μ M alpha-naphthylisothiocyanate and more than two hours of incubation. Glutathione depletion to as low as 30% of control was not toxic and lipid peroxidation did not appear to play a role as an increase in thiobarbituric acid-reactive species was not observed. The leakage of lactate dehydrogenase from cells is indicative of substantial membrane damage and increased permeability. The depletion of intracellular glutathione between 2 and 4 hours of incubation occurred in concert with the increase in lactate dehydrogenase leakage. Suggesting that this depletion is a function of membrane permeability and not due to the formation of glutathione conjugates. The data suggest further that 500 μ M alpha-naphthylisothiocyanate was sufficient to overwhelm the cells defense mechanisms allowing the toxic species to disrupt the cells ability to maintain membrane integrity. However, the nature of the toxic species is unknown, but may have been alpha-naphthylisothiocyanate itself, a metabolite or both.

Administration of alpha-naphthylisothiocyanate to laboratory animals can result in liver injury marked by

cholestasis, hyperbilirubinemia, necrosis of intralobular bile duct epithelium and focal necrosis of hepatocytes (Plaa and Priestly, 1977). The mechanism(s) by which alpha-naphthylisothiocyanate produces these effects is unknown. Inducers and inhibitors of mixed-function oxidase activity have been shown to potentiate or diminish alpha-naphthylisothiocyanate toxicity, respectively (Plaa and Priestly, 1976, El-Hawari and Plaa, 1979). These observations suggest that mixed-function oxidase metabolism to a toxic species is responsible. However, a different interpretation has been made suggesting that glutathione may be more important than mixed-function oxidase activity (Dahm and Roth, 1979). Recent investigations have demonstrated that depletion of hepatic glutathione protects against alpha-naphthylisothiocyanate-induced liver injury (Dahm and Roth, 1981).

This may be a direct effect on the excretion of alpha-naphthylisothiocyanate as a reversible glutathione conjugate into the bile. In this way influencing the amount of alpha-naphthylisothiocyanate reaching the bile ductule epithelium. The relationship between alpha-naphthylisothiocyanate conjugation, metabolism and transport at the cellular level is depicted in figure 25.

There is much to be learned before we will fully understand how alpha-naphthylisothiocyanate elicits its characteristic liver injury. The results obtained with isolated hepatocytes provide strong support for a potential role for glutathione conjugation and selective transport of alpha-naphthylisothiocyanate in alpha-naphthylisothiocyanate-induced liver injury.

Figure 25. Overview of Proposed Cycling of alpha-Naphthylisothiocyanate Within the Liver. ANIT = alpha-naphthylisothiocyanate, GS-ANIT = glutathione-alpha-naphthylisothiocyanate conjugate.

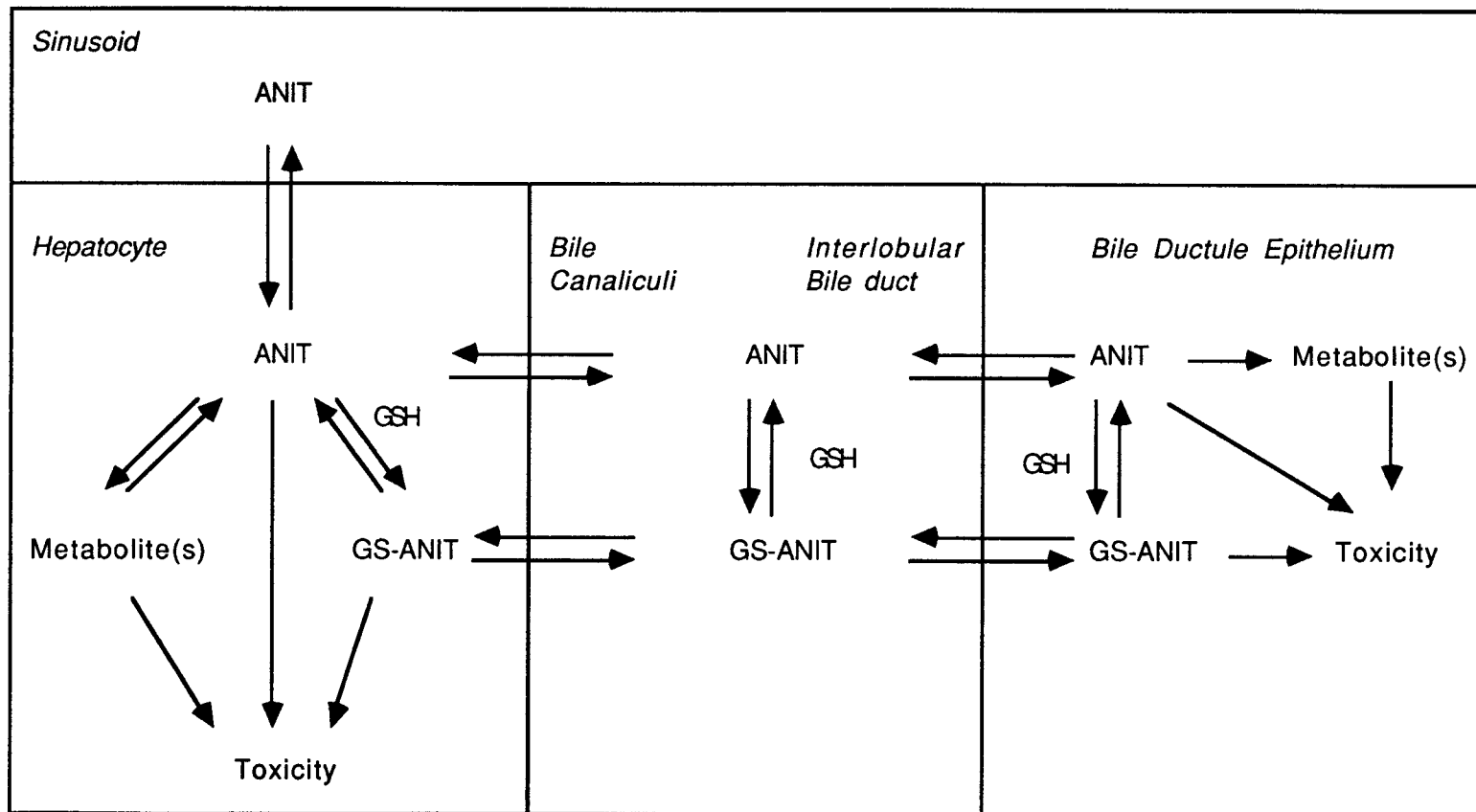


Figure 25.

SUMMARY AND FUTURE DIRECTIONS

Observations:

The investigations described in this thesis have provided valuable data and observations that are best summarized as follows.

S-(2-Chloroethyl)glutathione and S-(2-chloroethyl)-L-cysteine are direct-acting alkylating agents toward functional groups common to protein and nucleic acids with particularly high reactivity toward free sulfhydryl groups.

1,2-Dihaloethane-induced glutathione depletion in isolated rat hepatocytes is the result of its utilization in 1,2-dihaloethane metabolism. The amount and variety of glutathione-containing metabolites formed suggests that glutathione sulfur half-mustard formation is responsible for the majority of the 1,2-dibromoethane and 1-bromo-2-chloroethane-induced glutathione depletion.

The development of 1,2-dibromoethane-induced toxicity in isolated rat hepatocytes can be blocked by the maintenance of a relatively low amount of intracellular glutathione. Hepatocytes experience extensive lipid peroxidation concomitant with cell death following glutathione depletion. Lipid peroxidation is much involved in the development of cell death in this model system as indicated by the effects of butylated hydroxyanisole, desferal mesylate and N-acetyl-cysteine. However, the effects of ruthenium red suggest that lipid peroxidation may not be directly responsible for the 1,2-dibromoethane-induced cell death.

alpha-Naphthylisothiocyanate readily conjugates with glutathione to form a reversible S-conjugate. The depletion of hepatocellular glutathione following alpha-naphthyliso-

thiocyanate exposure is a consequence of this reversible conjugation coupled to the export of the glutathione conjugate, dissociation and re-uptake of alpha-naphthylisothiocyanate into the hepatocyte. This cyclic process results in the net transport of glutathione out of the hepatocyte.

Significance:

It was well established in the literature, prior to initiating my investigations, that two principle metabolic pathways were responsible for 1,2-dihaloethane metabolism. That, *in vivo*, the mixed-function oxidation pathway was thought responsible for the majority of 1,2-dihaloethane metabolism but that the direct conjugation with glutathione was responsible for the DNA alkylation, mutagenicity and carcinogenicity exhibited by these compounds. The contribution of either pathway in relationship to target organ toxicity was uncertain. In addition, protein alkylation was little studied in contrast to DNA alkylation.

My studies have demonstrated that the glutathione sulfur half-mustards should give rise to extensive protein alkylation as compared to the alkylation of DNA. Cysteinyl thiols being likely targets for alkylation in comparison to other amino acid functionalities.

The conjugation of glutathione with 1,2-dihaloethanes was determined to be responsible for much of the 1,2-dihaloethane-induced depletion of glutathione in isolated rat hepatocytes. This would give rise to the formation of a substantial amount of glutathione sulfur half-mustard concomitant with glutathione depletion. *In vivo*, 1,2-dihaloethane metabolism by the liver in this way may be responsible for the observed glutathione depletion and toxicity.

The importance of glutathione sulfur half-mustard formation in relationship to covalent binding and target organ toxicity remains unclear. My initial studies have demonstrated that 1,2-dibromoethane toxicity in isolated hepatocytes was dependent upon glutathione depletion and that lipid peroxidation was important in the expression of this toxicity.

The effects of alpha-naphthylisothiocyanate have been well documented with respect to the morphologic and physiologic conditions manifest in its cholestatic effect and hepatotoxicity. However, little is known about the mechanism(s) of action involved in eliciting the cholestatic and toxic effects. Various roles have been proposed for metabolites of alpha-naphthylisothiocyanate, tissue glutathione and polymorphonuclear leukocytes in alpha-naphthylisothiocyanate cholestasis and toxicity. Our studies with isolated rat hepatocytes and glutathione have clearly demonstrated that alpha-naphthylisothiocyanate conjugates reversibly with glutathione and that this conjugation may lead to glutathione depletion via alpha-naphthylisothiocyanate cycling. *In vivo*, this process may give rise to the targeted accumulation of alpha-naphthylisothiocyanate into the bile as a reversible glutathione conjugate. Resulting in the specific exposure of bile canalicular membranes and bile ductule epithelial cells, possibly accounting for some of the characteristic features in alpha-naphthylisothiocyanate-induced cholestasis and toxicity.

Recommendations for Future Research:

Based upon the results of my research and what is presently known about 1,2-dihaloethane toxicity, I would recommend that future research emphasize 1,2-dihaloethane

protein alkylation. Specifically, the identification of the alkylating species, sites of alkylation, identity of alkylated proteins and assessment of the importance of alkylation to protein structure and function. It is likely that these data will be of paramount importance in developing an understanding of the underlying mechanism of 1,2-dihaloethane toxicity.

In regards to alpha-naphthylisothiocyanate, the investigation of intra-hepatic glutathione homeostasis in relationship to the distribution of alpha-naphthylisothiocyanate should merit attention based on our results. The determination of the susceptibility of isolated intra-hepatic bile ductule epithelial cells would also be of interest. In addition, metabolites of alpha-naphthylisothiocyanate have been observed but not identified or characterized for their potential toxicologic effects.

For both 1,2-dihaloethanes and alpha-naphthylisothiocyanate there is a real need to more fully characterize the biochemical and morphologic changes within cells associated with their exposures. This would serve best in establishing the cause and effect relationship that is all important in understanding chemical-induced toxicity. In addition, this would greatly aid in determining specific sites of action leading to elucidation of the chemicals mechanism of action pertinent to the development of the toxic response.

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